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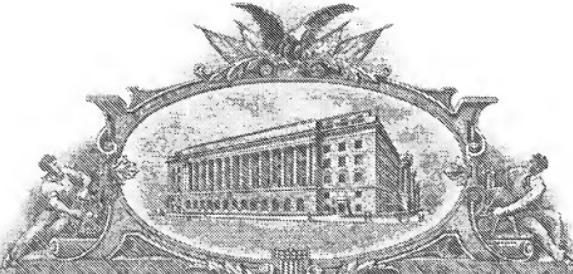
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET
 This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

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Respectfully submitted,

Signature

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PROVISIONAL APPLICATION FOR PATENT

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CONFORMATION SPECIFIC ANTIBODIES

BACKGROUND

Integrins are cell surface molecules that mediate important interactions between cells and between cells and the extracellular milieu. Integrins can adopt at least two different conformations on cell surfaces -- a non-activated conformation that does not bind to the integrin ligand and an activated conformation that can bind the integrin ligand. Cellular signalling can cause integrins to alter their conformation from a non-activated conformation to an activated conformation. After activation, integrins bind in a specific manner to their cognate ligands on the surface of other cells, in the extracellular matrix, or that are assembled in the clotting or complement cascades.

Each integrin includes an α subunit and a β subunit. Over twenty different integrin heterodimers are known. Many integrins are selectively expressed on particular cells in the body. For example, a subset of integrins are selectively expressed on leukocytes.

Integrins on leukocytes are of central importance in leukocyte emigration and in inflammatory and immune responses. Two exemplary integrins on leukocytes are LFA-1 and Mac-1. LFA-1 (α L β 2) binds to a number of cognate ligands, including inflammation-associated cell surface molecules (ICAM), e.g., ICAM-1, ICAM-2, ICAM-3, ICAM-4, and ICAM-5. Mac-1 (α M β 2) binds ICAM-1, the complement component iC3b, and the clotting component fibrinogen.

SUMMARY

Disclosed are binding proteins that interact with integrins ("integrin binding proteins, particularly specific conformations of integrins. An exemplary binding protein is an antibody. An integrin binding protein can preferentially interact with an activated conformation of an integrin, e.g., relative to a non-activated conformation, e.g., an inactive or resting conformation. An integrin binding protein can preferentially interact with a mimic of an activated conformation of an integrin (e.g., a modified integrin whose conformation is constrained in a state competent to bind to a cognate ligand), e.g., relative to a non-activated conformation, e.g., an inactive or resting conformation, or

mimics thereof. The integrin binding protein can bind with at least 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, 1000, or 10⁵ fold greater affinity to the preferred conformation relative to the disfavored conformation.

In one embodiment, the integrin binding protein can be used to modulate integrin activity, e.g., antagonize an activity of an activated integrin. For example, the integrin binding protein can be used to inhibit interaction between a cell that has an activated integrin on its surface and a cognate ligand of the activated integrin.

In one embodiment, the integrin binding protein interacts with a leukocyte integrin, e.g., LFA-1, e.g., activated LFA-1 (“aLFA-1”), e.g., human aLFA-1.

In one embodiment, the integrin binding protein is an antibody. The antibody can include one or more human regions, e.g., one or more human CDRs, one or more human frameworks (e.g., germline or somatically mutated human FR), or one or more human constant regions, or effectively human regions of the same.

In one embodiment, the integrin binding protein inhibits aLFA-1 activity. For example, the integrin binding protein prevents aLFA-1 from interacting with a binding partner, e.g., a cognate ligand of LFA-1. In particular cases, the antibody can prevent aLFA-1 from interacting with an ICAM, e.g., ICAM-1, ICAM-2, ICAM-3, ICAM-4, or ICAM-5.

The integrin binding protein can modulate (e.g., decrease) inflammation, and accordingly can be used to treat an inflammatory disorder. Accordingly, the integrin binding protein can be administered to a subject in an amount effective to treat or prevent such a disorder.

In one aspect, the invention features a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence. The HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1 (“aLFA-1”), e.g., with cation dependence (e.g., that detectably binds at 10 µg/ml protein concentration). For example, maximal binding requires the presence of a cation. The protein can require magnesium or manganese for binding to LFA-1. Exemplary cation concentrations is between 0.01 – 11mm, e.g., between 0.1 and 5 mM, or 0.1 and 3 mM. In one embodiment, the protein binds to LFA-1 in the presence of magnesium,

EGTA and the CBRLFA-1/2 antibody, but not in the presence of magnesium, calcium, and the CBRLFA-1/2 antibody.

In one embodiment, the protein can bind to a K287C/K294C I domain of α L. For example, the protein preferentially binds a K287C/K294C I domain of α L relative to L161C/F299C I domain of α L or wildtype α L.

In one embodiment, the protein binds aLFA-1 with a K_D of less than 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} , or 10^{-12} M. In one embodiment, the protein can reduce interaction between LFA-1 and a cognate ligand of LFA-1 (e.g., an ICAM, e.g., ICAM-1). In one embodiment, the protein can reduce interaction between a leukocyte and an ICAM-expressing cell, e.g., an endothelial cell.

In another aspect, the invention features a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1, wherein the heavy chain variable domain sequence includes (a) a CDR1 that includes at least 3, 4, or 5 amino acids (of 5) from RYVMW, (b) a CDR2 that includes at least 13, 14, 15, 16, or 17 amino acid (of 17) from YIWPSSGGNTYYADSVKG, and/or (c) a CDR3 that includes at least 8, 9, 10, 11 amino acids (of 11) from SYDFWSNAFDI. The protein can include other features described herein. In one embodiment, the protein includes features of D2-57, e.g., the CDR regions of the D2-57 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the D2-57 antibody.

In another aspect, the invention features a protein including an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1, wherein the light chain variable domain sequence includes (a) a CDR1 that includes at least 7, 8, 9, 10, or 11 amino acids (of 11) from RASQSIGSYLN, (b) a CDR2 that includes at least 4, 5, 6, or 7 amino acids (of 7) from AASSLQS, and/or (c) a CDR3 that includes at least 5, 6, 7, or 8 (of 8) amino acids from QQSYSTPS. The

protein can include other features described herein. In one embodiment, the protein includes features of D2-57, e.g., the CDR regions of the D2-57 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the D2-57 antibody.

In another aspect, the invention features a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1, wherein the heavy chain variable domain sequence includes (a) a CDR1 that includes at least 3, 4, or 5 amino acids (of 5) from HYGMS, (b) a CDR2 that includes at least 13, 14, 15, 16, or 17 amino acid (of 17) from VISPSGGRTLYADSVKG; and/or (c) a CDR3 that includes at least 5, 6, 7, or 8 amino acids (of 8) from HYSYAMDV. In one embodiment, the protein includes features of C1-54, e.g., the CDR regions of the C1-54 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the C1-54 antibody.

In another aspect, the invention features a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1, wherein the light chain variable domain sequence includes (a) a CDR1 that includes at least 7, 8, 9, 10, or 11 amino acids (of 11) from TASQSVDSNLA, (b) a CDR2 that includes at least 4, 5, 6, or 7 amino acids (of 7) from GASTRAT; and/or (c) a CDR3 that includes at least 6, 7, 8, 9, or 10 amino acids (of 10) from QQYNKWPPYS. In one embodiment, the protein includes features of C1-54, e.g., the CDR regions of the C1-54 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the C1-54 antibody.

In another aspect, the invention features an antibody that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin

light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1. The heavy chain variable domain sequence includes (a) a CDR1 that includes at least 3, 4, or 5 amino acids (of 5) from HYSMQ, (b) a CDR2 that includes at least 13, 14, 15, 16, or 17 amino acid (of 17) from YIGSSGGNTYYADSVKG, and/or (c) a CDR3 that includes at least 7, 8, 9, or 10 amino acids (of 10) from GTYNTSPFDY. In one embodiment, the protein includes features of P1-G10, e.g., the CDR regions of the P1-G10 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the P1-G10 antibody.

In another aspect, the invention features a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to an activated conformation of LFA-1. The light chain variable domain sequence includes (a) a CDR1 that includes at least 7, 8, 9, 10, or 11 amino acids (of 11) from SGDALGQKYAS, (b) a CDR2 that includes at least 4, 5, 6, or 7 amino acids (of 7) from QDSKRPS, and/or (c) a CDR3 that includes at least 5, 6, 7, 8, or 9 amino acids (of 9) from QAWDTTAYV. In one embodiment, the protein includes features of P1-G10, e.g., the CDR regions of the P1-G10 antibody. In one embodiment, the heavy and light chain variable domain sequences are at least 70, 80, 85, 90, 92, 93, 94, 95, 97, 98, 99, or 100% identical to corresponding variable domain sequences of the P1-G10 antibody.

A protein described herein can have at least 30, 50, 60, 70, 80, 90 or 100% of the CDR amino acid residues that are not identical to residues in the reference CDR sequences be identical to residues at corresponding positions in a human germline sequence. The protein can have at least 30, 50, 60, 70, 80, 90 or 100% of the FR regions be identical to FR sequence from a human germline sequence or a FR sequence of D2-57, C1-54, or P1-G10. Exemplary human germline sequences are provided below.

In another aspect, the invention features an antibody or a non-naturally occurring protein that preferentially binds to activated LFA-1 relative to inactivated LFA-1 and that competes with antibody D2-57, C1-54, or P1-G10 for binding to activated LFA-1.

In another aspect, the invention features an antibody or a non-naturally occurring protein that binds to an epitope that overlaps with an epitope recognized by antibody D2-57, C1-54, or P1-G10 on LFA-1, e.g., on activated LFA-1, or that binds to the same epitope as antibody D2-57, C1-54, or P1-G10.

In another aspect, the invention features a pharmaceutical composition that includes a protein described herein and a pharmaceutically acceptable salt. The invention also provides a kit that includes a protein described herein and instructions for therapeutic or diagnostic use.

In another aspect, the invention features a method of treating or preventing inflammation or an inflammatory disorder. The method includes: administering a protein described herein to a subject in an amount effective to treat or prevent the inflammation or the inflammatory disorder, e.g., to ameliorate at least one symptom of inflammation or the inflammatory disorder, or to delay the appearance of such symptom.

In one embodiment, the protein is administered at dosages less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks. For example, the recommended dose for the average patient can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks.

In one embodiment, the protein is administered at dosages effective to produce a detectable serum concentration whose mean trough concentration is less than 9, 8, 7, 6, 5, 4, 3, 2, 1 µg/ml. In one embodiment, the protein is administered in two phase, in which the first phase is characterized by administration of a first dose, and the second phase is characterized by administration of the second dose, different from the first dose. The first dose can be less than the second dose, or can be greater than the second dose, e.g., at least 20, 30, or 40% different.

For example, the first dose is an initial dose and, e.g., is less than 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg. The second dose can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg.

In one embodiment, the subject has psoriasis or is predisposed to psoriasis. For example, the subject has stable, plaque psoriasis. In one embodiment, the subject has psoriasis whose minimum body surface involvement is at least 2, 5, 10, 15, 20, or 25%.

In one embodiment, the protein is administered to a subject who has not been treated with another systemic therapy or with phototherapy, e.g., in the previous 30, 60, 90, or 180 days.

The protein can be administered at dosages effective to increase white blood cell count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%. The protein can be administered at dosages effective to increase eosinophils count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%.

In another aspect, the invention features a method of treating or preventing inflammation or an inflammatory disorder. The method includes administering a protein described herein to a subject in an amount effective to ameliorate inflammation or the inflammatory disorder, wherein the protein does not substantially interact with non-activated LFA-1 molecules in the subject.

In one embodiment, the protein is administered at dosages less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks. For example, the recommended dose for the average patient can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks.

In one embodiment, the protein is administered at dosages effective to produce a detectable serum concentration whose mean trough concentration is less than 9, 8, 7, 6, 5, 4, 3, 2, 1 µg/ml. In one embodiment, the protein is administered in two phase, in which the first phase is characterized by administration of a first dose, and the second phase is characterized by administration of the second dose, different from the first dose. The first dose can be less than the second dose, or can be greater than the second dose, e.g., at least 20, 30, or 40% different.

For example, the first dose is an initial dose and, e.g., is less than 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg. The second dose can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg.

In one embodiment, the subject has psoriasis or is predisposed to psoriasis. For example, the subject has stable, plaque psoriasis. In one embodiment, the subject has psoriasis whose minimum body surface involvement is at least 2, 5, 10, 15, 20, or 25%.

In one embodiment, the protein is administered to a subject who has not been treated with another systemic therapy or with phototherapy, e.g., in the previous 30, 60, 90, or 180 days.

The protein can be administered at dosages effective to increase white blood cell count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%. The protein can be administered at dosages effective to increase eosinophils count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%.

In another aspect, the invention features a method of treating or preventing an inflammation or an inflammatory disorder. The method includes: administering a protein described herein to a subject in an amount that is less than the amount required to treat or prevent inflammation or the inflammatory disorder using an antibody that does not preferentially bind to activated LFA-1 (e.g., binds to both activated and inactivated LFA-1 with substantially the same affinity, e.g., RAPTIVA®), wherein the protein does not substantially interact with non-activated LFA-1 molecules exposed on leukocytes of the subject.

In one embodiment, the protein is administered at dosages less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks. For example, the recommended dose for the average patient can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks. For example, the protein is administered at a dose less than RAPTIVA® to achieve substantially the same result.

In one embodiment, the protein is administered at dosages effective to produce a detectable serum concentration whose mean trough concentration is less than 9, 8, 7, 6, 5, 4, 3, 2, 1 µg/ml. In one embodiment, the protein is administered in two phase, in which the first phase is characterized by administration of a first dose, and the second phase is characterized by administration of the second dose, different from the first dose. The first dose can be less than the second dose, or can be greater than the second dose, e.g., at least 20, 30, or 40% different.

For example, the first dose is an initial dose and, e.g., is less than 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg. The second dose can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg.

In one embodiment, the subject has psoriasis or is predisposed to psoriasis. For example, the subject has stable, plaque psoriasis. In one embodiment, the subject has psoriasis whose minimum body surface involvement is at least 2, 5, 10, 15, 20, or 25%.

In one embodiment, the protein is administered to a subject who has not been treated with another systemic therapy or with phototherapy, e.g., in the previous 30, 60, 90, or 180 days.

The protein can be administered at dosages effective to increase white blood cell count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%. The protein can be administered at dosages effective to increase eosinophils count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%.

In another aspect, the invention features a method of treating or preventing an inflammation or an inflammatory disorder. The method includes: administering a protein described herein to a subject in an amount effective to ameliorate or delay appearance of at least one symptom of inflammation or the inflammatory disorder, wherein cells in the subject that do not present an activated LFA-1 protein on their surface are not targeted by the protein.

In one embodiment, the protein is administered at dosages less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks. For example, the recommended dose for the average patient can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg per week, e.g., for at least 2, 3, 5, 10, or 52 weeks.

In one embodiment, the protein is administered at dosages effective to produce a detectable serum concentration whose mean trough concentration is less than 9, 8, 7, 6, 5, 4, 3, 2, 1 μ g/ml. In one embodiment, the protein is administered in two phase, in which the first phase is characterized by administration of a first dose, and the second phase is characterized by administration of the second dose, different from the first dose. The first dose can be less than the second dose, or can be greater than the second dose, e.g., at least 20, 30, or 40% different.

For example, the first dose is an initial dose and, e.g., is less than 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg. The second dose can be less than 1, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.05, or 0.02 mg/kg.

In one embodiment, the subject has psoriasis or is predisposed to psoriasis. For example, the subject has stable, plaque psoriasis. In one embodiment, the subject has psoriasis whose minimum body surface involvement is at least 2, 5, 10, 15, 20, or 25%.

In one embodiment, the protein is administered to a subject who has not been treated with another systemic therapy or with phototherapy, e.g., in the previous 30, 60, 90, or 180 days.

The protein can be administered at dosages effective to increase white blood cell count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%. The protein can be administered at dosages effective to increase eosinophils count by at least 5, 10, 15, 20, 25, 30, 35, 40, or 45%.

For example, the subject has or is predisposed to a disorder that is caused at least in part by a T cell inflammatory response.

For example, the subject has or is predisposed to an inflammatory disorder selected from the group consisting of: allergic conditions such as eczema and asthma, Reiter's syndrome, HIV, cytokine-induced toxicity, transient hypogammaglobulinemia, malignancies (e.g., B-cell malignancies such as chronic lymphocytic leukemia or hairy cell leukemia), diseases involving leukocyte diapedesis, acute glomerulonephritis, asthma, immune deficiency disorders, invasion of tumor cells into secondary organs etc., insulinitis, atherosclerosis, conditions involving infiltration of T cells and chronic inflammatory responses, selective IgA deficiency, meningitis, chronic mucocutaneous , dermatoses with acute inflammatory components, sarcoidosis, skin hypersensitivity reactions (including poison ivy and poison oak), urticaria, nephrotic syndrome, acute appendicitis, inflammatory bowel disease (such as Crohn's disease and ulcerative colitis), encephalitis, wound healing, chronic obstructive pulmonary disease, myelothemia gravis, congenital X-linked infantile hypogammaglobulinemia, lupus, adult respiratory distress syndrome, orbital inflammatory disease, inflammatory breast disease, uveitis, psoriasis, HIV and rhinovirus infection, CNS inflammatory disorder, antigen-antibody complex mediated diseases, necrotizing enterocolitis, amyloidosis, thermal injury, bronchitis,

leukocyte adhesion deficiency II syndrome, autoimmune hemolytic anemia, peritonitis, pulmonary fibrosis, septic shock, multiple organ injury syndrome secondary to septicaemia or trauma, leukapheresis, pernicious anemia, nephritis, chronic bronchitis, common variable immunodeficiency, sclerodenna, glomerulonephritis, polymyositis, pelvic inflammatory disease, rhinitis, granulocyte transfusion associated syndromes, ulcerative colitis and Crohn's disease), viral infection, hemodialysis, autoimmune diseases (e.g., granulomatosis and vasculitis), lung inflammation, reactive arthritis, dermatitis, and leukocyte adhesion deficiency. Example of autoimmune disorders include: rheumatoid arthritis, systemic lupus erythematosus (SLE), diabetes mellitus, multiple sclerosis, Reynaud's syndrome, autoimmune thyroiditis, experimental autoimmune encephalomyelitis, Sjorgen's syndrome, juvenile onset diabetes, and immune responses associated with delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, and polymyositis,

In one embodiment, the protein is administered at a dosage that does not substantially increase risk for serious infection (e.g., no more than 0.4% of patients), risk for thrombocytopenia (e.g., no more than 0.3% of patients), risk for psoriasis aggravation (e.g., no more than 0.7% of patients), or risk of headache, chill, fever, nausea, myalgia, pain, arthritis, or arthralgia (e.g., no more than 32, 13, 7, 11, 8, 10, 0.4, and 0.3% of patients, respectively). In one embodiment, the protein can be the same frequency of side effects as RAPTIVA®, or less.

In another aspect, the invention features a method of suppressing an immune response. The method includes a protein described herein to a subject in an amount effective to suppress an immune response of the subject. In one embodiment, the subject has or is about to receive a transplant.

In another aspect, the invention features a method of treating or preventing a disorder in a subject. The method includes: identifying a subject in need of an anti-LFA-1 antibody that preferentially binds to the activated form of LFA-1, but which subject does not respond or tolerate an anti-LFA-1 antibody that binds to activated and non-activated LFA-1 protein with substantially the same affinity; and administering the anti-LFA-1 antibody that preferentially binds to the activated form of LFA-1, to the subject.

In another aspect, the invention features a method of modulating aLFA-1 activity. The method includes: providing an aLFA-1-binding protein of claim 1; and contacting the protein to aLFA-1, in an amount sufficient to modulate aLFA-1 activity.

For example, the contacting is in vitro or in vivo.

In one embodiment, the protein is contacted to aLFA-1 in the vicinity of a neoplastic cell (e.g., a cell found in laryngeal, epidermal, pulmonary, breast, renal, urothelial, colonic, prostatic, or hepatic cancer and/or metastasis). In one embodiment, the protein is contacted to aLFA-1 in the vicinity of an endothelial cell.

In another aspect, the invention features a method for detecting the presence of an aLFA-1 protein, in a sample, e.g., in vitro. The method includes: (i) contacting the sample (and optionally, a reference, e.g., control, sample) with an aLFA-1-binding protein described herein, under conditions that allow interaction of the aLFA-1-binding protein and the aLFA-1 protein to occur; and (ii) detecting interaction between the aLFA-1-binding protein, and the sample (and optionally, the reference, e.g., control, sample).

At least one of the aLFA-1 binding protein or the aLFA-1 is immobilized.

In another aspect, the invention features a method for detecting the presence of aLFA-1 (e.g., activated aLFA-1), e.g., in vivo. The method includes: (i) administering to a subject (and optionally a control subject) an aLFA-1-binding protein, under conditions that allow interaction of the aLFA-1-binding protein and the aLFA-1 protein to occur; and (ii) detecting location of the aLFA-1-binding protein in the subject or formation of a complex between the aLFA-1-binding protein and aLFA-1 in the subject. For example, the subject is a human subject. The detecting can include imaging the subject. For example, the aLFA-1-binding protein is labeled with an MRI detectable label.

The invention also includes a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence. The HC variable domain sequence and the LC variable domain sequence form an antigen binding site that detectably binds to both an integrin I domain in the activated conformation and an integrin I domain in the non-activated conformation, but preferentially binds to an integrin in the activated conformation relative to binding to the integrin in the non-activated conformation.

For example, the protein has at least a 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, or 1000 fold preference for binding to activated LFA-1 relative to inactivated LFA-1.

The protein can have at least a 1.5, 2, 3, 4, 5, or 10 preference for binding to activated LFA-1 relative to inactivated LFA-1, but no more than a 15, 20, 50, 70, 80, 100, 500, or 1000 fold preference.

The invention also includes a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence. The HC variable domain sequence and the LC variable domain sequence form an antigen binding site that detectably binds to both an integrin I domain in the open conformation and an integrin I domain in the closed conformation, but preferentially binds to the integrin I domain in the open conformation relative to the integrin I domain in the closed conformation.

For example, the protein has at least a 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, or 1000 fold preference for binding to activated LFA-1 relative to inactivated LFA-1.

The protein can have at least a 1.5, 2, 3, 4, 5, or 10 preference for binding to activated LFA-1 relative to inactivated LFA-1, but no more than a 15, 20, 50, 70, 80, 100, 500, or 1000 fold preference. In one embodiment, protein can bind to a disulfide-locked K287C/K294C I domain.

The invention also includes a protein that includes an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that detectably binds to both an integrin I domain of LFA-1 in the activated conformation and an integrin I domain in the non-activated conformation, but preferentially binds to activated LFA-1 relative to non-activated LFA-1. For example, the protein has at least a 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, or 1000 fold preference for binding to activated LFA-1 relative to inactivated LFA-1. The protein can have at least a 1.5, 2, 3, 4, 5, or 10 preference for binding to activated LFA-1 relative to inactivated LFA-1, but no more than a 15, 20, 50, 70, 80, 100,

500, or 1000 fold preference. In one embodiment, the I domain in the open conformation is a disulfide-locked K287C/K294C I domain.

Exemplary antibodies can include the following sequences or segments thereof:

Table 1: Exemplary Variable Domains

Name	Amino Acid Sequence
D2-57 LC	DIQMTQSPSSLSASVGDRVITTC RASQSIGSYLN WYQQKTGAKPALKIY AASSLQS GVPSRFGSGSGTDFTLTISSLQLEDFAVYYC QQSYSTPS FGQGTKVEIKRT
D2-57 HC	VQLLESGGGLVQPGGSLRLSCAASGFTFS RYVMW WVRQAPGKGLEWVS YIWPSSGGNTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAS SYDFWSNAFDI WGQGTMVTVSS
C1-54 LC	DIQMTQSPATLSVSPGERVTLSC TASQSVDSNLA WYQQKPGQAPRLLVY GASTRAT GVPARFSGSGSGTAFDTLTIDSLQSEDFAVYYC QQYNKWPPYS FGQGTKLEIKRT
C1-54 HC	VQLLESGGGLVQPGGSLRLSCAASGFTFS HYGMS WVRQAPGKGLEWVS VISPSSGGRTLYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK HYSYAMDV WGQGTTVTVSS
P1-G10 LC	SVLTQPPSVSVSPGQTASVTC SGDALGQKYAS WYQQKPGQSPVLVIF QDSKRPS GIPERFSGSNSGNTATLTISGTQAVDEADYYC QAWDTTAYV FGTGTKVTVL
P1-G10 HC	VQLLESGGGLVQPGGSLRLSCAASGFTFS HYSMQ WVRQAPGKGLEWVS YIGSSGGNTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR G.TYNTSPFDY WGQGTLVTVSS

An integrin binding antibody is typically monospecific, e.g., a monoclonal antibody, or antigen-binding fragment thereof. The aLFA-1-binding antibodies can be full-length (e.g., an IgG (e.g., an IgG1, IgG2, IgG3, IgG4), IgM, IgA (e.g., IgA1, IgA2), IgD, and IgE) or can include only an antigen-binding fragment (e.g., a Fab, F(ab')₂ or scFv fragment). The antibody, or antigen-binding fragment thereof, can include two heavy chain immunoglobulins and two light chain immunoglobulins, or can be a single chain antibody. The antibodies can, optionally, include a constant region chosen from a kappa, lambda, alpha, gamma, delta, epsilon or a mu constant region gene. An aLFA-1-binding antibody can include a heavy and light chain constant region substantially from a human antibody, e.g., a human IgG1 constant region or a portion thereof.

In one embodiment, the antibody is a recombinant or modified antibody, e.g., a chimeric, a humanized, a deimmunized, or an *in vitro* generated antibody. The term “recombinant” or “modified” antibody, as used herein, is intended to include all antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial antibody library, antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant antibodies include human, humanized, CDR grafted, chimeric, deimmunized, *in vitro* generated antibodies, and may optionally include framework and/or constant regions derived from human germline immunoglobulin-encoding nucleic acid sequences.

In one embodiment, the antibody binds to an epitope distinct from an epitope bound by known antibodies that bind to aLFA-1, e.g., distinct from an epitope bound by MHM23 (Hildreth et al., Eur. J. Immunol. 13:202-208 (1983)); M18/2 (IgG.sub.2a ; Sanches-Madrid et al., J. Exp. Med. 158:586 (1983)); H52 (American Type Culture Collection (ATCC) Deposit HB 10160); Mas191c and IOT18 (Vermot Desroches et al., Scand. J. Immunol. 33:277-286 (1991)); and NA-8 (WO 94/12214). In other embodiments, the antibody does not compete with known antibodies that bind to aLFA-1.

In still other embodiments, the antibody does not compete with an antibody described herein.

In one embodiment, the antibody binds to overlapping epitopes of, or competitively inhibits, the binding of an antibody disclosed herein to αLFA-1. In one embodiment, the antibody binds to an epitope that includes an amino acid that is within at least 12, 10, 8, 6, 5, or 3 amino acids of an epitope bound by an antibody described herein (e.g., D2-57, C1-54, or P1-G10). In one embodiment, the antibody includes an antigen binding site structure that recognizes one or more side chains that are positioned within 12, 10, 8, 6 or 4 Angstroms of an antibody described herein (e.g., D2-57, C1-54, or P1-G10). The epitope is generally in the extracellular region of LFA-1. The epitope can include one or more amino acid side chains on the α and/or β subunit. In one embodiment, the epitope includes one or more amino acid side chains on the I-domain of αL.

Further, any combination of αLFA-1-binding antibodies is within the scope of the invention, e.g., two or more antibodies that bind to different regions of αLFA-1, e.g., antibodies that bind to two different epitopes on the extracellular domain of αLFA-1, e.g., a bispecific antibody.

In one embodiment, the αLFA-1-binding antibody includes at least one light or heavy chain immunoglobulin (or two light chain immunoglobulins and two heavy chain immunoglobulins). Preferably, each immunoglobulin includes a light or a heavy chain variable region having at least one, two and, preferably, three complementarity determining regions (CDR's) substantially identical to a CDR from a light or heavy chain variable region, respectively, of an antibody described herein.

An integrin binding protein described herein can be used alone, e.g., can be administered to a subject or used *in vitro* in non-derivatized or unconjugated forms. In other embodiments, the integrin binding protein can be derivatized, modified or linked to another functional molecule, e.g., another polypeptide, protein, isotope, cell, or insoluble support. For example, the integrin binding protein can be functionally linked (e.g., by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities, such as an antibody (e.g., if the binding protein is an antibody, to form a bispecific or a multispecific antibody), a toxin, a label, a serum-residence

prolonging moiety (e.g. PEG), a therapeutic (e.g., a cytotoxic or cytostatic) agent or other moiety. An antibody can also be designed so that it can mediate complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC), or so that it does not mediate CDC or ADCC. For example, it can have a CDC- or ADCC-competent Fc domain, or a CDC- or ADCC-incompetent Fc domain.

In another aspect, the invention features a nucleic acid that includes a coding sequence that encodes a polypeptide including an immunoglobulin heavy chain variable domain sequence that binds to aLFA-1, e.g., an immunoglobulin heavy chain variable domain described herein. For example, the immunoglobulin heavy chain variable domain sequence can include: a CDR motif or CDR described herein. The immunoglobulin heavy chain variable domain sequence can include a framework region described herein. In one example, the variable domain sequence is a heavy chain variable domain is at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to an amino acid sequence described herein or a variable domain sequence thereof.

In another aspect, the invention features a nucleic acid that includes a coding sequence that encodes a polypeptide including an immunoglobulin light chain variable domain sequence that binds to aLFA-1, e.g., an immunoglobulin light chain variable domain described herein. For example, the immunoglobulin light chain variable domain sequence can include: a CDR motif or CDR described herein. The immunoglobulin light chain variable domain sequence can include a framework region described herein. In one example, the variable domain sequence is a light chain variable domain is at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to an amino acid sequence described herein or a variable domain sequence thereof.

A nucleic acid described herein can further include a promoter operably linked to the coding sequence. A nucleic acid can include a first and second coding sequence, e.g., wherein the first coding sequence encodes a polypeptide that includes an immunoglobulin heavy chain variable domain and the second coding sequence encodes a polypeptide that includes an immunoglobulin light chain variable domain.

In another aspect, the invention features a host cell that contains a first nucleic acid encoding a polypeptide including a heavy chain variable region and a second nucleic acid encoding a polypeptide including a light chain variable region. The heavy chain

variable region and the light chain variable region can associate to form an aLFA-1 binding protein. These variable regions can have one or more properties described herein, e.g., at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% identity to a sequence described herein, e.g., the sequence of a variable domain from an isolated antibody described herein or a human germline sequence described herein. The invention also includes a method of providing an aLFA-1-binding antibody. The method can include providing a host cell described herein; and expressing said first and second nucleic acids in the host cell under conditions that allow assembly of said light and heavy chain variable regions to form an antigen binding protein that interacts with aLFA-1.

In another aspect, the invention features a binding protein that includes a human or effectively human heavy chain immunoglobulin variable domain and a human or effectively human light chain immunoglobulin variable domain, wherein the binding protein binds to human aLFA-1. The protein can bind to aLFA-1 with a K_d of less than, 10^{-7} , 10^{-8} , 10^{-9} , or 10^{-10} M. The protein can include one or more additional features described herein.

In yet another aspect, the invention features a method of producing an aLFA-1-binding antibody, or antigen-binding fragment thereof. The method includes: providing a host cell that contains a first nucleic acid sequence encoding a polypeptide including a heavy chain variable region, e.g., a heavy chain variable region as described herein; providing a second nucleic acid sequence encoding a polypeptide including a light chain variable region, e.g., a light chain variable region as described herein; and expressing said first and second nucleic acid sequences in the host cell under conditions that allow assembly of said light and heavy chain variable regions to form an antigen binding protein that interacts with aLFA-1. The first and second nucleic acid sequences can be linked or unlinked, e.g., expressed on the same or different vector, respectively. The first and second nucleic acid sequences can be components of the same molecule or can reside on different molecules (e.g., different chromosomes or plasmids).

The host cell can be a eukaryotic cell, e.g., a mammalian cell, an insect cell, a yeast cell, or a prokaryotic cell, e.g., *E. coli*. For example, the mammalian cell can be a cultured cell or a cell line. Exemplary mammalian cells include lymphocytic cell lines (e.g., NSO), Chinese hamster ovary cells (CHO), COS cells, oocyte cells, and cells from

a transgenic animal, e.g., mammary epithelial cell. For example, nucleic acids encoding the antibodies described herein can be expressed in a transgenic animal. In one embodiment, the nucleic acids are placed under the control of a tissue-specific promoter (e.g., a mammary specific promoter) and the antibody is produced in the transgenic animal. For example, the antibody molecule is secreted into the milk of the transgenic animal, such as a transgenic cow, pig, horse, sheep, goat or rodent.

In another aspect, the invention features a method of treating or preventing an inflammatory disorder in a subject. The method includes providing an aLFA-1-binding protein, e.g. a protein described herein, and contacting the subject with the protein, in an amount sufficient to modulate or prevent the inflammatory disorder. The method can include identifying a subject as having or being at risk for having an inflammatory disorder.

The subject can be a mammal, e.g., a primate, preferably a higher primate, e.g., a human (e.g., a patient having, or at risk of, a disorder described herein).

The aLFA-1-binding protein can be administered to the subject systemically (e.g., orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, intranasally, transdermally, or by inhalation), topically, or by application to mucous membranes, such as the nose, throat and bronchial tubes.

The method can further include monitoring at least one indicator of inflammation, e.g., local temperature, swelling (e.g., as measured), redness, local or systemic white blood cell count, presence or absence of neutrophils, cytokine levels, elastase activity, and so forth. The subject can be monitored in one or more of the following periods: prior to beginning of treatment; during the treatment; or after one or more elements of the treatment have been administered. Monitoring can be used to evaluate the need for further treatment with the same aLFA-1-binding protein or other agents. A desired change in one or more of the parameters described above can be indicative of the improved condition of the subject. Information about the monitoring can be recorded, e.g., in electronic or digital form.

In another aspect, the invention features methods for detecting the presence of an aLFA-1 protein, in a sample, *in vitro* (e.g., a biological sample or a tissue biopsy). The subject method can be used to evaluate, e.g., diagnose or stage a disorder described

herein. The method includes: (i) contacting the sample (and optionally, a reference, e.g., control, sample) with an aLFA-1-binding protein, as described herein, under conditions that allow interaction of the aLFA-1-binding protein and the LFA-1 protein to occur; and (ii) detecting aLFA-1, e.g., by detecting formation of a complex between the LFA-1-binding protein and LFA-1, or by detecting an interaction between the aLFA-1-binding protein and LFA-1, in the sample (and optionally, the reference, e.g., control, sample). Formation of the complex can be indicative of the presence of aLFA-1 protein (e.g., activated aLFA-1 protein), and can indicate the suitability or need for a treatment described herein. For example, a statistically significant change in the formation of the complex in the sample relative to the reference sample, e.g., the control sample, is indicative of the presence of activated aLFA-1 in the sample.

In yet another aspect, the invention provides a method for detecting the presence of LFA-1 (e.g., activated aLFA-1) *in vivo* (e.g., *in vivo* imaging in a subject). The subject method can be used to evaluate, e.g., diagnose, localize, or stage a disorder described herein, e.g., inflammation, an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder. The method includes: (i) administering to a subject (and optionally a control subject) an aLFA-1-binding protein (e.g., an antibody or antigen binding fragment thereof), under conditions that allow interaction of the aLFA-1-binding protein and the aLFA-1 protein to occur; and (ii) detecting formation of a complex between the binding protein and aLFA-1, wherein a statistically significant change in the formation of the complex in the subject relative to the reference, e.g., the control subject or subject's baseline, is indicative of the presence of the aLFA-1. The presence of activated aLFA-1 in particular locations within a subject can be indicative of inflammation or an inflammatory disorder.

In other embodiments, a method of diagnosing or staging, a disorder as described herein (e.g., an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder), is provided. The method includes: (i) identifying a subject having, or at risk of having, the disorder; (ii) obtaining a sample of a tissue or cell affected with the disorder; (iii) contacting said sample or a control sample with an aLFA-1-binding protein, under conditions that allow interaction of the binding agent and the aLFA-1 protein to occur, and (iv) detecting formation of a complex. A statistically

significant increase in the formation of the complex between the binding protein and LFA-1 with respect to a reference sample, e.g., a control sample, is indicative of the disorder or the stage of the disorder. In one embodiment, the sample is obtained by non-surgical means, e.g., by a blood, saliva, or urine sample. In another embodiment, surgery is used.

Preferably, the aLFA-1-binding protein used in the *in vivo* and *in vitro* diagnostic methods is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound binding agent.

Although many embodiments of the disclosure are described in the context of binding proteins that preferentially bind to activated LFA-1 ("aLFA-1"), proteins that preferentially bind to a conformer of another target protein (e.g., another integrin, e.g., another leukocyte integrin subfamily member) or a different LFA-1 conformer can also be made and used.

Definitions

The term "binding protein" refers to a protein that can interact with a target molecule. An "integrin binding protein" refers to a protein that can interact with an integrin, and includes, in particular proteins that preferentially interact with an activated integrin, e.g., aLFA-1, or mimic thereof.

As used herein, the term "antibody" refers to a protein that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence. For example, an antibody can include a heavy (H) chain variable region (abbreviated herein as VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term "antibody" encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab fragments, F(ab')₂, a Fd fragment, a Fv fragments, and dAb fragments) as well as complete antibodies.

The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., *et al.*

(1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al.

(1987) J. Mol. Biol. 196:901-917). Kabat definitions are used herein. Each VH and VL is typically composed of three CDR's and four FR's, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

An "immunoglobulin domain" refers to a domain from the variable or constant domain of immunoglobulin molecules. Immunoglobulin domains typically contain two β -sheets formed of about seven β -strands, and a conserved disulphide bond (see, e.g., A. F. Williams and A. N. Barclay 1988 *Ann. Rev Immunol.* 6:381-405).

As used herein, an "immunoglobulin variable domain sequence" refers to an amino acid sequence which can form the structure of an immunoglobulin variable domain. For example, the sequence may include all or part of the amino acid sequence of a naturally-occurring variable domain. For example, the sequence may omit one, two or more N- or C-terminal amino acids, internal amino acids, may include one or more insertions or additional terminal amino acids, or may include other alterations. In one embodiment, a polypeptide that includes immunoglobulin variable domain sequence can associate with another immunoglobulin variable domain sequence to form a target binding structure (or "antigen binding site"), e.g., a structure that preferentially interacts with an activated integrin structure or a mimic of an activated integrin structure, e.g., relative to an non-activated structure.

The VH or VL chain of the antibody can further include all or part of a heavy or light chain constant region, to thereby form a heavy or light immunoglobulin chain, respectively. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light chain constant region includes a CL domain. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. The term "antibody" includes intact

immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof). The light chains of the immunoglobulin may be of types kappa or lambda. In one embodiment, the antibody is glycosylated. An antibody can be functional for antibody-dependent cytotoxicity and/or complement-mediated cytotoxicity.

One or more regions of an antibody can be human or effectively human. For example, one or more of the variable regions can be human or effectively human. For example, one or more of the CDRs can be human, e.g., HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3. Each of the light chain CDRs can be human. HC CDR3 can be human. One or more of the framework regions can be human, e.g., FR1, FR2, FR3, and FR4 of the HC or LC. In one embodiment, all the framework regions are human, e.g., derived from a human somatic cell, e.g., a hematopoietic cell that produces immunoglobulins or a non-hematopoietic cell. In one embodiment, the human sequences are germline sequences, e.g., encoded by a germline nucleic acid. One or more of the constant regions can be human or effectively human. In another embodiment, at least 70, 75, 80, 85, 90, 92, 95, or 98% of, or the entire antibody can be human or effectively human.

All or part of an antibody can be encoded by an immunoglobulin gene or a segment thereof. Exemplary human immunoglobulin genes include the kappa, lambda, alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH--terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g., gamma (encoding about 330 amino acids).

The term "antigen-binding fragment" of a full length antibody (or simply "antibody portion," or "fragment"), as used herein, refers to one or more fragments of a full-length antibody that retain the ability to specifically bind to a target of interest. Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include (i) a Fab fragment, a monovalent fragment consisting of

the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) that retains functionality. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules known as single chain Fv (scFv). See *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883.

Antibody fragments can be obtained using any appropriate technique including conventional techniques known to those with skill in the art. The term "monospecific antibody" refers to an antibody that displays a single binding specificity and affinity for a particular target, *e.g.*, epitope. This term includes a "monoclonal antibody" or "monoclonal antibody composition," which as used herein refer to a preparation of antibodies or fragments thereof of single molecular composition. As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by heavy chain constant region genes.

An "effectively human" immunoglobulin variable region is an immunoglobulin variable region that includes a sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. An "effectively human" antibody is an antibody that includes a sufficient number of human amino acid positions such that the antibody does not elicit an immunogenic response in a normal human.

A "humanized" immunoglobulin variable region is an immunoglobulin variable region that is modified to include a sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. Descriptions of "humanized" immunoglobulins include, for example, US 6,407,213 and US 5,693,762.

As used herein, “binding affinity” refers to the apparent association constant or K_a . The K_a is the reciprocal of the dissociation constant (K_d). A binding protein may, for example, have a binding affinity of at least 10^{-5} , 10^{-6} , 10^{-7} or 10^{-8} M for a particular target molecule. Higher affinity binding of a binding ligand to a first target relative to a second target can be indicated by a higher K_a (or a smaller numerical value K_d) for binding the first target than the K_a (or numerical value K_d) for binding the second target. In such cases the binding protein has specificity for the first target (e.g., a protein in a first conformation or mimic thereof) relative to the second target (e.g., the same protein in a second conformation or mimic thereof). Differences in binding affinity (e.g., for specificity or other comparisons) can be at least 1.5, 2, 3, 4, 5, 10, 15, 20, 50, 70, 80, 100, 500, 1000, or 10^5 fold.

Binding affinity can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (e.g., using a fluorescence assay). Exemplary conditions for evaluating binding affinity are in PBS (phosphate buffered saline) at pH 7.2 at 30°C. These techniques can be used to measure the concentration of bound and free binding protein as a function of binding protein (or target) concentration. The concentration of bound binding protein ([Bound]) is related to the concentration of free binding protein ([Free]) and the concentration of binding sites for the binding protein on the target where (N) is the number of binding sites per target molecule by the following equation:

$$[\text{Bound}] = N \cdot [\text{Free}] / ((1/K_a) + [\text{Free}]).$$

It is not always necessary to make an exact determination of K_a , though, since sometimes it is sufficient to obtain a quantitative measurement of affinity, e.g., determined using a method such as ELISA or FACS analysis, is proportional to K_a , and thus can be used for comparisons, such as determining whether a higher affinity is, e.g., 2-fold higher, to obtain a qualitative measurement of affinity, or to obtain an inference of affinity, e.g., by activity in a functional assay, e.g., an *in vitro* or *in vivo* assay.

An “isolated composition” refers to a composition that is removed from at least 90% of at least one component of a natural sample from which the isolated composition can be obtained. Compositions produced artificially or naturally can be “compositions of

at least" a certain degree of purity if the species or population of species of interests is at least 5, 10, 25, 50, 75, 80, 90, 92, 95, 98, or 99% pure on a weight-weight basis.

An "epitope" refers to the site on a target compound that is bound by a binding protein (e.g., an antibody such as a Fab or full length antibody). In the case where the target compound is a protein, the site can be entirely composed of amino acid components, entirely composed of chemical modifications of amino acids of the protein (e.g., glycosyl moieties), or composed of combinations thereof. Overlapping epitopes include at least one common amino acid residue.

Calculations of "homology" or "sequence identity" between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The optimal alignment is determined as the best score using the GAP program in the GCG software package with a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences.

In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 92%, 95%, 97%, 98%, or 100% of the length of the reference sequence. For example, the reference sequence may be the length of the immunoglobulin variable domain sequence.

As used herein, the term "substantially identical" (or "substantially homologous") is used herein to refer to a first amino acid or nucleic acid sequence that contains a sufficient number of identical or equivalent (e.g., with a similar side chain, e.g.,

conserved amino acid substitutions) amino acid residues or nucleotides to a second amino acid or nucleic acid sequence such that the first and second amino acid or nucleic acid sequences have (or encode proteins having) similar activities, e.g., a binding activity, a binding preference, or a biological activity. In the case of antibodies, the second antibody has the same specificity and has at least 50% of the affinity relative to the same antigen.

Sequences similar or homologous (e.g., at least about 85% sequence identity) to the sequences disclosed herein are also part of this application. In some embodiment, the sequence identity can be about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. In addition, substantial identity exists when the nucleic acid segments hybridize under selective hybridization conditions (e.g., highly stringent hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, which is incorporated by reference. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: (1) low stringency hybridization conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for low stringency conditions); (2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; (3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and (4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. Very high stringency conditions (4) are the preferred conditions and the ones that should be used unless otherwise specified. The invention includes nucleic acids that hybridize with low, medium, high, or very high stringency to a nucleic acid described herein or to a complement thereof, e.g., nucleic

acids encoding a binding protein described herein. The nucleic acids can be the same length or within 30, 20, or 10% of the length of the reference nucleic acid. The nucleic acid can correspond to a region encoding an immunoglobulin variable domain sequence.

An integrin binding protein may have mutations relative to a binding protein described herein (e.g., a conservative or non-essential amino acid substitutions), which do not have a substantial effect on the protein functions. Whether or not a particular substitution will be tolerated, i.e., will not adversely affect biological properties, such as binding activity can be predicted, e.g., using the method of Bowie, et al. (1990) *Science* 247:1306-1310.

A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). It is possible for many framework and CDR amino acid residues to include one or more conservative substitutions.

Consensus sequences for biopolymers can include positions which can be varied among various amino acids. For example, the symbol “X” in such a context generally refers to any amino acid (e.g., any of the twenty natural amino acids or any of the nineteen non-cysteine amino acids). Other allowed amino acids can also be indicated for example, using parentheses and slashes. For example, “(A/W/F/N/Q)” means that alanine, tryptophan, phenylalanine, asparagine, and glutamine are allowed at that particular position.

A “non-essential” amino acid residue is a residue that can be altered from the wild-type sequence of the binding agent, e.g., the antibody, without abolishing or more preferably, without substantially altering a biological activity, whereas an “essential” amino acid residue results in such a change.

The terms “polypeptide” or “peptide” (which may be used interchangeably) refer to a polymer of three or more amino acids linked by a peptide bond, e.g., between 3 and 30, 12 and 60, or 30 and 300, or over 300 amino acids in length. The polypeptide may include one or more unnatural amino acids. Typically, the polypeptide includes only natural amino acids. A “protein” can include one or more polypeptide chains.

Accordingly, the term “protein” encompasses polypeptides. A protein or polypeptide can also include one or more modifications, e.g., a glycosylation, amidation, phosphorylation, and so forth. The term “small peptide” can be used to describe a polypeptide that is between 3 and 30 amino acids in length, e.g., between 8 and 24 amino acids in length.

The term “cognate ligand” refers to a naturally occurring ligand of an integrin, including naturally occurring variants thereof (e.g., splice variants, naturally occurring mutants, and isoforms).

The term “mimic,” in the context of a mimic of a conformation of an integrin or portion thereof, refers to a modified integrin which has a bias for at least one particular conformation relative to a naturally occurring integrin, or portion thereof.

Statistical significance can be determined by any art known method. Exemplary statistical tests include: the Students T-test, Mann Whitney U non-parametric test, and Wilcoxon non-parametric statistical test. Some statistically significant relationships have a P value of less than 0.05 or 0.02. Particular binding proteins may show a difference, e.g., in specificity or binding, that are statistically significant (e.g., P value < 0.05 or 0.02). The terms “induce”, “inhibit”, “potentiate”, “elevate”, “increase”, “decrease” or the like, e.g., which denote distinguishable qualitative or quantitative differences between two states, and may refer to a difference, e.g., a statistically significant difference, between the two states.

Other features and advantages of the instant invention will become more apparent from the following detailed description and claims. Embodiments of the invention can include any combination of features described herein. The contents of all references, patent applications (published and unpublished) and published patents, cited throughout this application are hereby expressly incorporated by reference. This application also incorporates by reference the 2003 FDA-approved product label for RAPTIVA®.

DETAILED DESCRIPTION

The invention provides, *inter alia*, binding proteins (e.g., antibodies) that bind to an integrin in an activated conformation, e.g., activated LFA-1 ("aLFA-1"), e.g., relative to a non-activated conformation of LFA-1. In one embodiment, the binding proteins inhibit at least one function of an aLFA-1, e.g., inhibit a binding interaction between aLFA-1 and a cognate ligand of aLFA-1, e.g., an ICAM protein. The binding proteins can be used to treat or prevent an inflammatory disorder or other disorder described herein.

LFA1

Lymphocyte function-associated antigen-1 (LFA-1) is a member of the leukocyte integrin subfamily. LFA-1 is a heterodimer of an integrin alpha subunit, α L (CD11a), and a beta subunit β 2 (CD18).

Other integrins of the leukocyte integrin subfamily also include the β 2 subunit (CD 18), but have distinct alpha subunits. For example, MAC-1 is a heterodimer of β 2 and α M (CD11b). p150.95 is a heterodimer of β 2 and α X (CD11c). Springer, T A (1990) *Nature* 346:425-433; Larson, R S and Springer T A, (1990) *Immunol Rev* 114:181-217; Van der Vieren, M et al. (1995) *Immunity* 3:683-690. (Springer, T A (1990) *Nature* 346:425-433; Larson, R S and Springer T'A, (1990) *Immunol Rev* 114:181-217; Van der Vieren, M et al. (1995) *Immunity* 3:683-690). The leukocyte integrins mediate a wide range of adhesive interactions that are essential for normal immune and inflammatory responses.

An exemplary amino acid sequence of the α subunit of human LFA-1 (α L) is as follows:

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>gi|4504757|ref|NP_002200.1| integrin alpha L precursor; antigen CD11A  
(p180), lymphocyte function-associated antigen 1, alpha polypeptide from  
Homo sapiens
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MKDSCITVMAMALLSGFFFFPAssYNLDVGRGARSFSPPRAGRHFYRVLQVGNGVIVGAPGEGNSTGSL  
YCCQSGTGCHLPVTLRGNSNTSKYLGMLTADPTDGSILACDPGLSRTCDQNTYLSGLCYLFQRQLNQGP  
LQGRPGFQECIKGNVNDLVFLFDGMSLSPDEFQKILDFMKDVMKKLNSTSYYQFAAVQFSTSYYKTEFDFSD  
YVKWKPDAALLKHVKHMLLTNTPGAINYVATEVFREELARPDAVKLIIITDGEATDSGNIDAAKDII  
RYIIGIGKHQTKEQSQTLLHKPASKPASEFVKILDTFEKLKDFTELQKKIYVIEGTSKQDLTSFNMLESL  
SSGISAIDLSRGHAVVGAVGAKDWGGFLDKADLQDDTFIGNEPLTPEVRAGYLGYTVTLPSRQKTSLL  
ASGAPFRQHMGRVLLFQEPQGGGHWSQVQTIHGTQIGSYFGGELCGVVDQDGETELLIIGAPLFYQEQR  
GGRVFIYQRQLGFEEVS ELQGDPGYPLGRGEAITALTDINGDGLVDVAVGAPEEQGAVYIIFNGRHGG  
LSPQPSQRRIEGTQVLSGIQWFGRSIHGVIQLEGDGGLADVAVGAEQMIVLSSRPVVDMVTMSFPABIP
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VHEVECSYSTSNSMKKEGVNINITCFQIKSLYPQFQGRLVANLITYTLQLDGHRTRRGLFPGGRHIELRRNIA
 VTTSMSCDFSFHFPVQCVLDLSPINVLSFLSWEEECTPRDQRQAQGKDIPPLRPSLHSSETWEIPIFFKN
 CGEDKKCBEANLRVSFSPARSRLRALTAFAISLSEVLSNLEEDAYWVQLDLHPPGLSPRKVEMLKPHSQ
 IIPVSCEELPEESRLLSRALSCNVSSPIFKAGHSVALQMFMNTLVNNSWGSVELHANVTCNNEEDSLLLED
 NSATTIPIILYPIPINLILQDQEDSTLYVSFTPKGPKIHQVKHMVYQVRIOPSIHDHNIPTEAVVGPQPSPS
 EGPITHQWSVQMEPPVPCHYEDLERLPDAEPCPLPGALPRCPVFVFRQELIVQVIGTLELVEIASSMFS
 LCSSLISPNSSXHFHLYGSNASLAQVMKVDVVYEKQMLLYVLSGIGGLLIFIVLYKGFFKRN
 LKEKMEAGRGPVPNQIAPEDSEQLASQEBADPGPCLKPLHEKDSESGGGD

An exemplary amino acid sequence of the β subunit of human LFA-1 ($\beta 2$) is as follows:

>gi|4557886|ref|NP_000202.1| integrin beta chain, beta 2 precursor;
 Integrin, beta-2 (antigen CD18 (p95), lymphocyte function-associated;
 cell surface adhesion glycoprotein (LFA-1/CR3/P150, 959 beta subunit
 precursor) from *Homo sapiens*

MLGLRPPLALVGLLSLGCVLSQECTKFKVSSCRECIESGPGCTWCQKLNFTGPGDPDSIRCCTRQPLLM
 RGCAADDIMDPTSLAETQEDHNGQQKQLSPQKVTLYLRRPGQAAAFNVTFRRAKGYPIDLYVLMDSLVSYML
 DDLRNVKLGDDLLRALNEITESGRIGFGSVFDKTVLPFVNTHPDKLRNCPKKEKECQCQPFARHVKL
 TNNSNQFQTEVGKQNLSDAEGPGLDAMMQVAACPEEIVGRNVRTLLVFATDDGFHFAFGDKLGAILT
 PNDGRCHLLEDNLVYKRSNEDFPGVQGLAHKLAENNIQIPFATTSRVMVKTYEKLTEIIPKSAVGELESDDSS
 NVVHLIKNAYNKLSRVFDLHDNALPDTLKVTYDSCNSGVTHRNRQPRGDCDGQVINPVITFQVKVTATEC
 1QEQSFCVIRALFDIVTUVQVLPCCECRDQSRSRDLSCHGKGFLECGICRCRDTGYIGKNCCECTQGRSS
 QLEQSGCRKDNNSIICSGLGEDCVCQGCLCHTSVDPGKLIYQGYCECGCTINCERYNGQVCGGPGRGLFCFG
 KCRCHPGFEGSACQCERTTEGCLNPRRVECSGRGRCRCRNCECHSGYQLPLCQECPGCPSPCGKYISCAE
 CLKFEKGPFGKNCASACPGLQLSNPNPVKGRTCKERDSEGCWVAYTLEQQDGMDRYLIVYDERECVAQGP
 IAAIVGTVAGIVLIGILLVIKALIHLSDLREYRPFKEKLKSQWNNDNPLFKSATTTVMNPKAES

Proteins that preferentially bind to an activated leukocyte integrin can be used to modulate a leukocyte activity and a physiological activity mediated by a leukocyte, e.g., an activated leukocyte. Such binding proteins can be used to modulate (e.g., inhibit) leukocyte migration, leukocyte adherence, or inflammation.

Integrins can adopt a plurality of conformations, including an activated and a non-activated conformation. Additional conformational intermediates are also available. The conformation of an integrin can be biased, for example, by modifying the amino acid sequence of the integrin. A bias in conformation can be introduced within a single domain of an integrin, e.g., within an integrin I domain, a β -propeller domain, or between domains, or between subunits. In one embodiment, the integrin is modified by the engineering of an intra-molecular or inter-molecular disulfide bond. Modified integrin molecules can be used as mimics of a conformation of a naturally occurring integrin.

The N-terminal region of the integrin α subunits contains seven repeats of about 60 amino acids each, and has been predicted to fold into a 7-bladed β -propeller domain

(Springer, T A (1997) *Proc Natl Acad Sci USA* 94:65-72). The leukocyte integrin α subunits (such as the $\alpha 1$, $\alpha 2$, $\alpha 10$, $\alpha 11$, αL , αM , αD , αX , and αE subunits) contain an inserted domain or I-domain of about 200 amino acids (Larson, R S et al. (1989) *J Cell Biol* 108:703-712; Takada, Y et al. (1989) *EMBO J* 8:1361-1368; Briesewitz, R et al. (1993) *J Biol Chem* 268:2989-2996; Shaw, S K et al. (1994) *J Biol Chem* 269:6016-6025; Camper, L et al. (1998) *J Biol Chem* 273:20383-20389). The I-domain is predicted to be inserted between β -sheets 2 and 3 of the β -propeller domain. The three dimensional structure of the αM , αL , $\alpha 1$ and $\alpha 2$ I-domains has been solved and shows that it adopts the dinucleotide-binding fold with a unique divalent cation coordination site designated the metal ion-dependent adhesion site (MIDAS) (Lee, J-O, et al. (1995) *Structure* 3:1333-1340; Lee, J -O, et al. (1995) *Cell* 80:631-638; Qu, A and Leahy, D J (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, D J (1996) *Structure* 4:931-942; Emsley, J et al. (1997) *J Biol Chem* 272:28512-28517; Baldwin, E T et al. (1998) *Structure* 6:923-935; Kallen, J et al. (1999) *J Mol Biol* 292:1-9). The C-terminal region of the αM subunit has been predicted to fold into a β -sandwich structure (Lu, C et al. (1998) *J Biol Chem* 273:15138-15147).

US 2002-0123614 describes, *inter alia*, exemplary methods for obtaining and using conformationally biased integrin molecules. In one embodiment, an integrin is locked in a particular conformation using a disulfide bond. Computational algorithms for designing and/or modeling protein conformations are described, for example, in WO 98/47089. The SSBOND program (Hazes, B and Dijkstra, B W (1988) *Protein Engineering* 2:119-125) can be used to identify positions where disulfide bonds can be introduced in a protein structure by mutating appropriately positioned pairs of residues to cysteine.

Disulfide bond formation can occur between two cysteine residues that are appropriately positioned within the three-dimensional structure of a protein. Accordingly, a protein can be stabilized in a desired conformation by introducing at least one cysteine substitution into the amino acid sequence such that a disulfide bond is formed. The introduction of a single cysteine substitution is performed in circumstances in which an additional cysteine residue is present in the native amino acid sequence of the protein at an appropriate position such that a disulfide bond is formed. More commonly, two

cysteine substitutions are introduced into the amino acid sequence of the protein at positions that allow a disulfide bond to form, thereby stabilizing the protein in a desired conformation. In another embodiment, the distance between the C β . carbons of the residues that are substituted for cysteine is 3.00-8.09 Å. In yet another embodiment, the distance between the C β carbons in the disulfide bond is in the range of 3.41-7.08 Å.

Typically, cysteine substitutions are introduced such that the formation of a disulfide bond is favored only in one protein conformation, such that the protein is stabilized in that particular conformation. Cysteine substitutions can be produced by mutagenesis of DNA encoding the polypeptides of interest (e.g., integrin polypeptides). For example, an isolated nucleic acid molecule encoding a modified integrin I-domain polypeptide can be created by introducing one or more nucleotide substitutions into the nucleotide sequence of an integrin gene such that one or more codons, e.g., cysteine codons, are introduced into the encoded protein. Mutations can be introduced into a nucleic acid sequence by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

Additional methods for obtaining and using integrins in a locked conformation are described, e.g., in Shimaoka, M et al. (2003) *Cell* 112,99-111 ; Shimaoka, M et al. (2002) *Annu. Rev. Biophys. Biomol. Struct.* 31,485-516; and Shimaoka, M et al. (2001) *Proc Natl Acad Sci U S A* 98:6009-6014. Luo et al. (2003) *Proc Natl Acad Sci U S A*, 100(5):2403-8 describe a conformationally biased integrin in which a glycan moiety is used to alter conformational preference. Luo et al. *J Biol Chem.* 2003 Dec 16 (Epub ahead of print), PMID: 14681220, describe additional conformationally biased, e.g., disulfide locked conformations.

For example, a conformationally biased integrin can include a modified integrin I-domain that is biased towards (e.g., locked in) the open conformation or a closed conformation. The open conformation may bind to a cognate ligand of the integrin with high affinity.

A disulfide locked molecule can be produced from a nucleic acid sequence that includes at least one codon substitution that inserts one or more (e.g., two) cysteine codons. The codons can be positions such that in the encoded protein, the distance between the C β carbons of the residues that are substituted for cysteines is in the range of

3.00-8.09 Angstroms e.g., as predicted by protein modeling. In a further embodiment, the distance between the C β carbons in the disulfide bond is in the range of 3.41-7.08 Angstroms.

Examples of integrin I-domains that are conformationally biased towards a particular conformation, e.g., an active "open" conformation, or a non-activated "closed" conformation include the following. The α L K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, L161C/T300C, and L289C/K294C mutants, and the α M Q163C/Q309C and D294C/Q31 IC mutants are stabilized in "open" conformations that bind the cognate ligand with high or intermediate affinity, whereas the α L L289C/K294C mutant and the α M Q163C/R313C mutants are stabilized in non-activated "closed" conformations that do not bind to the cognate ligand. The affinity of E284C/E301C for the cognate ligand is nearly comparable to that of K287C/K294C, e.g., high-affinity. The affinity of L161C/F299C, K160C/F299C, and L161C/T300C for the cognate ligand are significantly higher than wild-type, but 20-30 times lower than high-affinity α L I-domain, K287C/K294C. L161C/F299C, K160C/F299C, and L161C/T300C are referred to herein as intermediate affinity α L I-domains.

The I-domain of α L is described as follows, with secondary structure information below:

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1  GNVDLVFLF DGSMSLQPDE FQKILDPMKD VMKKLSNTSY QFAAVQFSTS
     EEEEEEE E BTTS HHH HHHHHHHHHHH HHHHTTSSE EEEEEEEESSS

50 YKTEFDPSDY VKRKDPDALL KHVKHMLLLT NTFGAINYVA TEVFREELGA
    EEESB HHHH HHHTTHHHHT SS      B      HHHHHHHHHHH HHTTGCGGT

100 RPDATKVLI ITDGEATDSG NIDAAKDIIR YIIGIGKHFQ TKESQETLHK
    TTSEEEEEE EE S   S      GGGTTSEE EEEE SS      STTTGGGGTT

150 FASKPASEFV KILDTEFEKLK DLFTTELQKKI
    TS SSHHHHE EETTTTTTTT TTT

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See, e.g., PDB structures: (1) 1MQA “Crystal Structure Of High Affinity Alphal I Domain In The Absence Of Ligand Or Metal” (mmdbId:21776); (2) 1MQ9 “Crystal Structure Of High Affinity Alphal I Domain With Ligand Mimetic Crystal Contact” (mmdbId:21775); (3) 1MQ8 “Crystal Structure Of Alphal I Domain In Complex With Icam-1” (mmdbId:21774); and (4) 1MJN “Crystal Structure Of The Intermediate Affinity Al I Domain Mutant” (mmdbId:21755).

Conformationally biased integrin molecules may include just a modified integrin I-domain from an integrin α subunit, or the entire mature α subunit extracellular domain, or the entire mature α subunit, and/or may be further associated with an integrin β subunit extracellular domain and/or entire subunit. In one embodiment, a modified integrin I-domain polypeptide is a soluble protein, e.g., a heterodimeric soluble protein, or a monomeric soluble protein.

A model of the I-like domain of the integrin β -subunit that is supported by experimental data (Huang, C et al. (2000) J Biol Chem 275:21514-24) has also been made. The data confirm the location of the key C-terminal α -helix that undergoes the dramatic 10 Angstrom conformational movement in I domains. The I and I-like domains align well in this region.

Identification of aLFA-1 binding proteins

A number of methods can be used to identify proteins that bind to aLFA-1 and other active integrins. Many of these methods use conformationally-biased integrin proteins as targets.

One exemplary method for identifying antibodies that bind to aLFA-1 includes immunizing a non-human animal with a conformationally biased LFA-1 protein or a conformationally biased domain thereof. Spleen cells can be isolated from the immunized animal and used to produce hybridoma cells using standard methods. In one embodiment, the non-human animal includes one or more human immunoglobulin genes.

Another exemplary method for identifying proteins that bind to aLFA-1 includes: providing a library of proteins and selecting from the library one or more proteins that bind to a conformationally biased molecule, e.g., a conformationally biased integrin, e.g.,

aLFA-1. The selection can be performed in a number of ways. For example, the library can be provided in the format of a display library or a protein array. Prior to selecting, the library can be pre-screened (e.g., depleted) to remove members that interact with a non-target molecule, e.g., an LFA-1 molecule in the non-activated conformation.

The conformationally biased target molecule can be tagged and recombinantly expressed. In one embodiment, the conformationally biased target molecule is purified and attached to a support, e.g., to affinity beads, or paramagnetic beads or other magnetically responsive particles.

A conformationally biased target molecule can also be expressed on the surface of a cell. Members of the display library that specifically bind to the cell can be selected. It is also possible to use an endogenous or other wildtype form of an integrin. For example, members of the display library that specifically bind to a cell, only if the integrin is activated, can be selected.

Display Libraries

In one embodiment, a display library is used to identify proteins that bind to an integrin in an activated conformation, e.g., aLFA-1. A display library is a collection of entities; each entity includes an accessible protein component (e.g., a Fab or scFv) and a recoverable component (e.g., a nucleic acid) that encodes or identifies the protein component. The protein component can be of any length, e.g. from three amino acids to over 300 amino acids. In a selection, the protein component of each member of the library is probed with a conformationally biased integrin protein and if the protein component binds to the protein, the display library member is identified, e.g., by retention on a support. The protein component can include one or more immunoglobulin variable domains or variants of another domain. Methods using immunoglobulin domains for display are described below (see, e.g., "Antibody Display Libraries").

Retained display library members are recovered from the support and analyzed. The analysis can include amplification and a subsequent selection under similar or dissimilar conditions. For example, positive and negative selections can be alternated. The analysis can also include determining the amino acid sequence of the protein component and purification of the protein component for detailed characterization.

A variety of formats can be used for display libraries. Examples include the following.

Phage Display. One format utilizes viruses, particularly bacteriophages. This format is termed “phage display.” The protein component is typically covalently linked to a bacteriophage coat protein. The linkage results from translation of a nucleic acid encoding the protein component fused to the coat protein. The linkage can include a flexible peptide linker, a protease site, or an amino acid incorporated as a result of suppression of a stop codon. Phage display is described, for example, in Ladner *et al.*, U.S. Patent No. 5,223,409; Smith (1985) *Science* 228:1315-1317; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; de Haard *et al.* (1999) *J. Biol. Chem.* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20; Hoogenboom *et al.* (2000) *Immunol Today* 2:371-8; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Rebar *et al.* (1996) *Methods Enzymol.* 267:129-49; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982.

Phage display systems have been developed for filamentous phage (phage f1, fd, and M13) as well as other bacteriophage (e.g. T7 bacteriophage and lambdoid phages; see, e.g., Santini (1998) *J. Mol. Biol.* 282:125-135; Rosenberg *et al.* (1996) *Innovations* 6:1-6; Houshmet al. (1999) *Anal Biochem* 268:363-370). The filamentous phage display systems typically use fusions to a minor coat protein, such as gene III protein, and gene VIII protein, a major coat protein, but fusions to other coat proteins such as gene VI protein, gene VII protein, gene IX protein, or domains thereof can also be used (see, e.g., WO 00/71694). In one embodiment, the fusion is to a domain of the gene III protein, e.g., the anchor domain or “stump,” (see, e.g., U.S. Patent No. 5,658,727 for a description of the gene III protein anchor domain). It is also possible to physically associate the protein being displayed to the coat using a non-peptide linkage, e.g., a non-covalent bond or a non-peptide covalent bond. For example, a disulfide bond and/or c-

fos and c-jun coiled-coils can be used for physical associations (see, e.g., Crameri *et al.* (1993) *Gene* 137:69 and WO 01/05950).

Bacteriophage displaying the protein component can be grown and harvested using standard phage preparatory methods, e.g. PEG precipitation from growth media. After selection of individual display phages, the nucleic acid encoding the selected protein components can be isolated from cells infected with the selected phages or from the phage themselves, after amplification. Individual colonies or plaques can be picked, the nucleic acid isolated and sequenced.

Cell-based Display. In still another format the library is a cell-display library. Proteins are displayed on the surface of a cell, e.g., a eukaryotic or prokaryotic cell. Exemplary prokaryotic cells include *E. coli* cells, *B. subtilis* cells, and spores (see, e.g., Lu *et al.* (1995) *Biotechnology* 13:366). Exemplary eukaryotic cells include yeast (e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Hansenula*, or *Pichia pastoris*). Yeast surface display is described, e.g., in Boder and Wittrup (1997) *Nat. Biotechnol.* 15:553-557 and WO 03/029456.

In one embodiment, diverse nucleic acid sequences are cloned into a vector for yeast display. The cloning joins the variegated sequence with a domain (or complete) yeast cell surface protein, e.g., Aga2, Aga1, Flo1, or Gas1. A domain of these proteins can anchor the polypeptide encoded by the variegated nucleic acid sequence by a transmembrane domain (e.g., Flo1) or by covalent linkage to the phospholipid bilayer (e.g., Gas1). The vector can be configured to express two polypeptide chains on the cell surface such that one of the chains is linked to the yeast cell surface protein. For example, the two chains can be immunoglobulin chains.

In one embodiment, nucleic acids encoding immunoglobulin heavy chains that have been mutagenized based on an initial aLFA-1-binding immunoglobulin are introduced into yeast cells of one cell type, and nucleic acids encoding immunoglobulin light chains that have been mutagenized based on an initial aLFA-1-binding immunoglobulin are introduced into yeast cells of the other cell type. These two populations of cells can be combined to form diploid yeast that each express an immunoglobulin heavy and light chain. The yeast cells can be selected and/or screened for cells that bind to aLFA-1, e.g., bind with improved affinity.

Ribosome Display. RNA and the polypeptide encoded by the RNA can be physically associated by stabilizing ribosomes that are translating the RNA and have the nascent polypeptide still attached. Typically, high divalent Mg²⁺ concentrations and low temperature are used. See, e.g., Mattheakis *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:9022 and Hanes *et al.* (2000) *Nat Biotechnol.* 18:1287-92; Hanes *et al.* (2000) *Methods Enzymol.* 328:404-30; and Schaffitzel *et al.* (1999) *J Immunol Methods.* 231(1-2):119-35.

Protein-Nucleic Acid Fusions. Another format utilizes protein-nucleic acid fusions. Protein-nucleic acid fusions can be generated by the in vitro translation of mRNA that include a covalently attached puromycin group, e.g., as described in Roberts and Szostak (1997) *Proc. Natl. Acad. Sci. USA* 94:12297-12302, and U.S. Patent No. 6,207,446. The mRNA can then be reverse transcribed into cDNA and crosslinked to the protein.

Other Display Formats. Yet another display format is a non-biological display in which the protein component is attached to a non-nucleic acid tag that identifies the protein component. For example, the tag can be a chemical tag attached to a bead that displays the protein or a radiofrequency tag (see, e.g., U.S. Patent No. 5,874,214).

Epitope Specific Binding proteins. Display technology can also be used to obtain binding proteins, e.g., antibodies, that bind to particular epitopes of a target. Epitopes can be classified as "conformational" or "sequential". Conformational epitopes involve amino-acid residues that have a defined relative orientation in a properly folded target even though the amino acids may be substantially separated in the sequence (e.g., separated by at least one, two, four, six, eight or ten amino acids). Sequential epitopes involve short portions of the polypeptide chain that bind an antibody whatever the folding state of the protein (e.g., native or unfolded). Binding proteins for conformational epitopes can be identified, for example, by using competing non-target molecules that lack the particular epitope or are mutated within the epitope, e.g., with alanine. Such non-target molecules can be used in a negative selection procedure as described below, as competing molecules when binding a display library to the target, or as a pre-elution agent, e.g., to capture in a wash solution dissociating display library members that are not specific to the target. In another implementation, epitope specific binding proteins are

identified by eluting display library members with a competing binding protein that binds to the epitope of interest on the target molecule. Binding proteins that bind sequential epitopes can be selected, for example, using short peptides that have amino-acid sequences found in a target protein. Often binding proteins that bind to conformational epitopes also bind weakly to one or another peptide that contains some of the amino acids involved in the conformational epitope. Thus, one can select for binding to a peptide at very low stringency and then select for binding to the folded target protein.

Affinity Maturation. In one embodiment, a binding protein that binds to a target is modified, e.g., by mutagenesis, to provide a pool of modified binding proteins. The modified binding proteins are then evaluated to identify one or more altered binding proteins which have altered functional properties (e.g., improved binding, improved stability, lengthened stability *in vivo*). In one implementation, display library technology is used to select or screen the pool of modified binding proteins. Higher affinity binding proteins are then identified from the second library, e.g., by using higher stringency or more competitive binding and washing conditions. Other screening techniques can also be used.

In some implementations, the mutagenesis is targeted to regions known or likely to be at the binding interface. If, for example, the identified binding proteins are antibodies, then mutagenesis can be directed to the CDR regions of the heavy or light chains as described herein. Further, mutagenesis can be directed to framework regions near or adjacent to the CDRs, e.g., framework regions, particular within ten, five, or three amino acids of a CDR junction.. In the case of antibodies, mutagenesis can also be limited to one or a few of the CDRs, e.g., to make step-wise improvements.

In one embodiment, mutagenesis is used to make an antibody more similar to one or more germline sequences. One exemplary germlining method can include: identifying one or more germline sequences that are similar (e.g., most similar in a particular database) to the sequence of the isolated antibody. Then mutations (at the amino acid level) can be made in the isolated antibody, either incrementally, in combination, or both. For example, a nucleic acid library that includes sequences encoding some or all possible germline mutations is made. The mutated antibodies are then evaluated, e.g., to identify an antibody that has one or more additional germline residues relative to the isolated

antibody and that is still useful (e.g., has a functional activity). In one embodiment, as many germline residues are introduced into an isolated antibody as possible.

In one embodiment, mutagenesis is used to substitute or insert one or more germline residues into a CDR region. For example, the germline CDR residue can be from a germline sequence that is similar (e.g., most similar) to the variable region being modified. After mutagenesis, activity (e.g., binding or other functional activity) of the antibody can be evaluated to determine if the germline residue or residues are tolerated. Similar mutagenesis can be performed in the framework regions.

Selecting a germline sequence can be performed in different ways. For example, a germline sequence can be selected if it meets a predetermined criteria for selectivity or similarity, e.g., at least a certain percentage identity, e.g., at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.5% identity. The selection can be performed using at least 2, 3, 5, or 10 germline sequences. In the case of CDR1 and CDR2, identifying a similar germline sequence can include selecting one such sequence. In the case of CDR3, identifying a similar germline sequence can include selecting one such sequence, but may include using two germline sequences that separately contribute to the amino-terminal portion and the carboxy-terminal portion. In other implementations more than one or two germline sequences are used, e.g., to form a consensus sequence.

In one embodiment, with respect to a particular reference variable domain sequence, e.g., a sequence described herein, a related variable domain sequence has at least 30, 40, 50, 60, 70, 80, 90, 95 or 100% of the CDR amino acid positions that are not identical to residues in the reference CDR sequences, residues that are identical to residues at corresponding positions in a human germline sequence (i.e., an amino acid sequence encoded by a human germline nucleic acid).

In one embodiment, with respect to a particular reference variable domain sequence, e.g., a sequence described herein, a related variable domain sequence has at least 30, 50, 60, 70, 80, 90 or 100% of the FR regions are identical to FR sequence from a human germline sequence, e.g., a germline sequence related to the reference variable domain sequence.

Accordingly, it is possible to isolate an antibody which has similar activity to a given antibody of interest, but is more similar to one or more germline sequences,

particularly one or more human germline sequences. For example, an antibody can be at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5% identical to a germline sequence in a region outside the CDRs (e.g., framework regions). Further an antibody can include at least 1, 2, 3, 4, or 5 germline residues in a CDR region, the germline residue being from a germline sequence of similar (e.g., most similar) to the variable region being modified. Germline sequences of primary interest are human germline sequences. The activity of the antibody (e.g., the binding activity) can be within a factor of 100, 10, 5, 2, 0.5, 0.1, and 0.001 of the original antibody. An exemplary germline sequences include VKI-O2, VL2-1, VKIII-L2::JK2, vg3-23, V3-23::JH4, and V3-23::JK6.

Some exemplary mutagenesis techniques include: error-prone PCR (Leung *et al.* (1989) *Technique* 1:11-15), recombination (see, e.g., USSN 10/279,633), DNA shuffling using random cleavage (Stemmer (1994) *Nature* 389-391; termed “nucleic acid shuffling”), RACHITT™ (Coco *et al.* (2001) *Nature Biotech.* 19:354), site-directed mutagenesis (Zoller *et al.* (1987) *Nucl Acids Res* 10:6487-6504), cassette mutagenesis (Reidhaar-Olson (1991) *Methods Enzymol.* 208:564-586) and incorporation of degenerate oligonucleotides (Griffiths *et al.* (1994) *EMBO J* 13:3245).

In one example of affinity maturation the methods described herein are used to first identify a binding protein from a display library that binds an αLFA-1 with at least a minimal binding specificity for a target or a minimal activity, e.g., an equilibrium dissociation constant for binding of less than 1 nM, 10 nM, or 100 nM. The nucleic acid sequence encoding the initial identified binding protein are used as a template nucleic acid for the introduction of variations, e.g., to identify a second binding protein that has enhanced properties (e.g., binding affinity, kinetics, or stability) relative to the initial binding protein. Alternatively, the amino-acid sequence of one or more CDRs can be used as a guide for design of a nucleic acid library that includes nucleic acids encoding the isolated sequence and many neighboring sequences. Such diversified nucleic acids can be introduced into a display vector containing the initial isolate and improved variants are selected from the library.

Off-Rate Selection. Since a slow dissociation rate can be predictive of high affinity, particularly with respect to interactions between polypeptides and their targets,

the methods described herein can be used to isolate binding proteins with a desired kinetic dissociation rate (i.e. reduced) for a binding interaction to a target.

To select for slow dissociating binding proteins from a display library, the library is contacted to an immobilized target. The immobilized target is then washed with a first solution that removes non-specifically or weakly bound biomolecules. Then the immobilized target is eluted with a second solution that includes a saturation amount of free target, i.e., replicates of the target that are not attached to the particle. The free target binds to biomolecules that dissociate from the target. Rebinding is effectively prevented by the saturating amount of free target relative to the much lower concentration of immobilized target.

The second solution can have solution conditions that are substantially physiological or that are stringent. Typically, the solution conditions of the second solution are identical to the solution conditions of the first solution. Fractions of the second solution are collected in temporal order to distinguish early from late fractions. Later fractions include biomolecules that dissociate at a slower rate from the target than biomolecules in the early fractions.

Further, it is also possible to recover display library members that remain bound to the target even after extended incubation. These can either be dissociated using chaotropic conditions or can be amplified while attached to the target. For example, phage bound to the target can be contacted to bacterial cells.

Selecting and Screening for Specificity. "Selection", in the context of a display library, refers to a process in which many members of a display library are allowed to contact the target and those that bind are recovered and propagated. The selection can be from a library having numerous members, e.g., more than 10^{10} members. "Screening", in the context of a display library, refers to a process in which isolated members of the library are tested singly for binding to the target. Through automation, thousands of candidates may be screened in a highly parallel process. The display library selection methods described herein can include a selection process that discards display library members that bind to a non-target molecule.

Examples of non-target molecules, e.g., for an LFA-1 binding antibody, include, e.g., integrins other than LFA-1. In another example, for an aLFA-1 binding antibody,

e.g., an antibody that preferentially binds to activated LFA-1, the non-target molecule can be an LFA-1 molecule in a conformation other than activated, e.g., a non-activated conformation.

In one implementation, a so-called “negative selection” step is used to discriminate between the target and related non-target molecule and a related, but distinct non-target molecule. The display library or a pool thereof is contacted to the non-target molecule. Members of the sample that do not bind the non-target are collected and used in subsequent selections for binding to the target molecule or even for subsequent negative selections. The negative selection step can be prior to or after selecting library members that bind to the target molecule.

In another implementation, a screening step is used. After display library members are isolated for binding to the target molecule, each isolated library member is tested for its ability to bind to a non-target molecule (e.g., a non-target listed above). For example, a high-throughput ELISA screen can be used to obtain this data. The ELISA screen can also be used to obtain quantitative data for binding of each library member to the target. The non-target and target binding data are compared (e.g., using a computer and software) to identify library members that specifically bind to aLFA-1.

The display library selection and screening methods described herein can include a selection or screening process that selects for display library members that bind to specific sites on the target molecule. For example, elution with high concentration of an antibody described herein selects for phage that bind to the epitope bound by such an antibody. One can screen for a phage that binds to a particular epitope of aLFA-1 by performing ELISAs with and without a competing antibody that recognizes the epitope in the buffer.

Diversity

Display libraries and other libraries include variation at one or more positions in the displayed protein component. The variation at a given position can be synthetic or natural. For some libraries, both synthetic and natural diversity are included.

Synthetic Diversity. Libraries can include regions of diverse nucleic acid sequence that originate from artificially synthesized sequences. Typically, these are

formed from degenerate oligonucleotide populations that include a distribution of nucleotides at each given position. The inclusion of a given sequence is random with respect to the distribution. One example of a degenerate source of synthetic diversity is an oligonucleotide that includes NNN wherein N is any of the four nucleotides in equal proportion.

Synthetic diversity can also be more constrained, e.g., to limit the number of codons in a nucleic acid sequence at a given trinucleotide to a distribution that is smaller than NNN. For example, such a distribution can be constructed using less than four nucleotides at some positions of the codon. In addition, trinucleotide addition technology can be used to further constrain the distribution.

So-called “trinucleotide addition technology” is described, e.g., in Wells *et al.* (1985) *Gene* 34:315-323, US 4,760,025 and US 5,869,644. Oligonucleotides are synthesized on a solid phase support, one codon (i.e., trinucleotide) at a time. The support includes many functional groups for synthesis such that many oligonucleotides are synthesized in parallel. The support is first exposed to a solution containing a mixture of the set of codons for the first position. The unit is protected so additional units are not added. The solution containing the first mixture is washed away and the solid support is deprotected so a second mixture containing a set of codons for a second position can be added to the attached first unit. The process is iterated to sequentially assemble multiple codons. Trinucleotide addition technology enables the synthesis of a nucleic acid that at a given position can encode a number of amino acids. The frequency of these amino acids can be regulated by the proportion of codons in the mixture. Further the choice of amino acids at the given position is not restricted to quadrants of the codon table as is the case if mixtures of single nucleotides are added during the synthesis. Synthetic oligonucleotides including randomized or spiked codons can be also be used for producing a library for an affinity maturation selection.

Natural Diversity. Libraries can include regions of diverse nucleic acid sequence that originate (or are synthesized based on) from different naturally-occurring sequences. An example of natural diversity that can be included in a display library is the sequence diversity present in immune cells (see also below). Nucleic acids are prepared from these immune cells and are manipulated into a format for protein display.

Antibody Display Libraries

In one embodiment, the display library presents a diverse pool of proteins, each of which includes at least one and typically two immunoglobulin variable domains. Display libraries are particularly useful, for example, for identifying human or effectively human antibodies that recognize human antigens. Since the constant and framework regions of the antibody are human, these therapeutic antibodies may avoid themselves being recognized and targeted as antigens. The constant regions are also optimized to recruit effector functions of the human immune system. The *in vitro* display selection process surmounts the inability of a normal human immune system to generate antibodies against self-antigens.

A typical antibody display library displays a protein that includes a VH domain and a VL domain. The display library can display the antibody as a Fab fragment (e.g., using two polypeptide chains) or a single chain Fv (e.g., using a single polypeptide chain). Other formats can also be used.

As in the case of the Fab and other formats, the displayed antibody can include a constant region as part of a light or heavy chain. In one embodiment, each chain includes one constant region, e.g., as in the case of a Fab. In other embodiments, additional constant regions are displayed.

Antibody libraries can be constructed by a number of processes (see, e.g., de Haard *et al.* (1999) *J. Biol. Chem.* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20, and Hoogenboom *et al.* (2000) *Immunol Today* 21:371-8). Further, elements of each process can be combined with those of other processes. The processes can be used such that variation is introduced into a single immunoglobulin domain (e.g., VH or VL) or into multiple immunoglobulin domains (e.g., VH and VL). The variation can be introduced into an immunoglobulin variable domain, e.g., in the region of one or more of CDR1, CDR2, CDR3, FR1, FR2, FR3, and FR4, referring to such regions of either and both of heavy and light chain variable domains. In one embodiment, variation is introduced into all three CDRs of a given variable domain. In another preferred embodiment, the variation is introduced into CDR1 and CDR2, e.g., of a heavy chain variable domain. Any combination is feasible. In one process, antibody libraries are constructed by inserting diverse oligonucleotides that encode CDRs into the

corresponding regions of the nucleic acid encoding the display protein or portion thereof. The oligonucleotides can be synthesized using a variety of subunits, e.g., monomeric nucleotides or trinucleotides. For example, Knappik *et al.* (2000) *J. Mol. Biol.* 296:57-86 describe a method for constructing CDR encoding oligonucleotides using trinucleotide synthesis and a template with engineered restriction sites for accepting the oligonucleotides.

In another process, an animal, e.g., a rodent, is immunized with the aLFA-1. The animal is optionally boosted with the antigen to further stimulate the response. Then spleen cells are isolated from the animal, and nucleic acid encoding VH and/or VL domains is amplified and cloned for expression in the display library.

In yet another process, antibody libraries are constructed from nucleic acid amplified from naïve germline immunoglobulin genes (e.g., human genes). The amplified nucleic acid includes nucleic acid encoding the VH and/or VL domain. Sources of immunoglobulin-encoding nucleic acids are described below. Amplification can include PCR, e.g., with primers that anneal to the conserved constant region, or another amplification method.

Nucleic acid encoding immunoglobulin domains or fragments thereof can be obtained from the immune cells of, e.g., a human, a primate, mouse, rabbit, camel, or rodent. Cells can be selected for a particular property. For example, B cells at various stages of maturity, including naïve B cells, can be selected.

Fluorescent-activated cell sorting (FACS) can be used to sort B cells that express surface-bound IgM, IgD, or IgG molecules. Further, B cells expressing different isotypes of IgG can be isolated. B and T cells can be cultured and stimulated *in vitro*, e.g., by culturing with feeder cells or by adding mitogens or other modulatory reagents, such as antibodies to CD40, CD40 ligand or CD20, phorbol myristate acetate, bacterial lipopolysaccharide, concanavalin A, phytohemagglutinin or pokeweed mitogen.

Cells can also be isolated from a subject that has an immunological disorder, e.g., systemic lupus erythematosus (SLE), rheumatoid arthritis, vasculitis, Sjogren syndrome, systemic sclerosis, or anti-phospholipid syndrome. The subject can be a human, or an animal, e.g., an animal model for the human disease, or an animal having an analogous

disorder. Cells can be isolated from a transgenic non-human animal that includes a human immunoglobulin locus.

The cells can have activated a program of somatic hypermutation. Cells can be stimulated to undergo somatic mutagenesis of immunoglobulin genes, for example, by treatment with anti-immunoglobulin, anti-CD40, and anti-CD38 antibodies (see, e.g., Bergthorsdottir *et al.* (2001) *J Immunol.* 166:2228). In another embodiment, the cells are naïve.

The nucleic acid encoding an immunoglobulin variable domain can be isolated from a natural repertoire by the following exemplary method. First, RNA is isolated from the immune cell. Full length (i.e., capped) mRNAs are separated (e.g. by dephosphorylating uncapped RNAs with calf intestinal phosphatase). The cap is then removed with tobacco acid pyrophosphatase and reverse transcription is used to produce the cDNAs.

The reverse transcription of the first (antisense) strand can be done in any manner with any suitable primer. See, e.g., de Haard *et al.* (1999) *J. Biol. Chem.* 274:18218-30. The primer binding region can be constant among different immunoglobulins, e.g., in order to reverse transcribe different isotypes of immunoglobulin. The primer binding region can also be specific to a particular isotype of immunoglobulin. Typically, the primer is specific for a region that is 3' to a sequence encoding at least one CDR. In another embodiment, poly-dT primers may be used (and may be preferred for the heavy-chain genes).

A synthetic sequence can be ligated to the 3' end of the reverse transcribed strand. The synthetic sequence can be used as a primer binding site for binding of the forward primer during PCR amplification after reverse transcription. The use of the synthetic sequence can obviate the need to use a pool of different forward primers to fully capture the available diversity.

The variable domain-encoding gene is then amplified, e.g., using one or more rounds. If multiple rounds are used, nested primers can be used for increased fidelity. The amplified nucleic acid is then cloned into a display library vector.

Any method for amplifying nucleic acid sequences may be used for amplification. Methods can be used that maximize and do not bias diversity. A variety of techniques

can be used for nucleic acid amplification. The polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,195 and 4,683,202, Saiki, *et al.* (1985) *Science* 230, 1350-1354) utilizes cycles of varying temperature to drive rounds of nucleic acid synthesis. Transcription-based methods utilize RNA synthesis by RNA polymerases to amplify nucleic acid (U.S. Pat. No 6,066,457; U.S. Pat. No 6,132,997; U.S. Pat. No 5,716,785; Sarkar *et. al.*, *Science* (1989) 244: 331-34 ; Stofler *et al.*, *Science* (1988) 239: 491). NASBA (U.S. Patent Nos. 5,130,238; 5,409,818; and 5,554,517) utilizes cycles of transcription, reverse-transcription, and RNaseH-based degradation to amplify a DNA sample. Still other amplification methods include rolling circle amplification (RCA; U.S. Patent Nos. 5,854,033 and 6,143,495) and strand displacement amplification (SDA; U.S. Patent Nos. 5,455,166 and 5,624,825).

Secondary Screening Methods

Display libraries can be used to select candidate display library members that bind to the target. Each such candidate library member or any candidate αLFA-1 binding protein can be further analyzed, e.g., to further characterize its binding properties for the target. Each candidate display library member can be subjected to one or more secondary screening assays. The assay can be for a binding property, a physiological property (e.g., cytotoxicity, renal clearance, immunogenicity), a structural property (e.g., stability, conformation, oligomerization state) or another functional property (e.g., ability to modulate an activity of an integrin-expressing cell, e.g., a leukocyte, or ability to modulate inflammation or an inflammation associated- response). The same assay can be used repeatedly, but with varying conditions, e.g., to determine pH, ionic, or thermal sensitivities.

As appropriate, the assays can use the display library member directly, a recombinant polypeptide produced from the nucleic acid encoding a displayed polypeptide, a synthetic peptide synthesized based on the sequence of a displayed polypeptide. In the case of a candidate αLFA-1 binding protein from any source, the protein can be obtained, e.g., from such a source or by recombinant production. Exemplary assays for binding properties include the following.

Exemplary Biological Assays

Candidate aLFA-1 binding proteins can be evaluated for their activity *in vitro* (e.g., in a cell-free or cell-based system) or *in vivo* (e.g., in an animal model described below). For example, the proteins can be evaluated for their ability to inhibit an activity of LFA-1 expressing cells, e.g., a binding activity of an LFA-1 expressing cell. In another example, the proteins can be evaluated for their ability to target cells that present activated LFA-1.

The binding of LFA-1 expressing cells to a cognate ligand can be evaluated, e.g., using cellular assays. ICAM-1 is expressed, e.g., on leukocytes, endothelium, and dermal fibroblasts (Dustin et al., *J. Immunol.* 137: 245-254 (1986)), ICAM-2 expressed on resting endothelium and lymphocytes (de Fougerolles et al., *J. Exp. Med.* 174: 253-267 (1991)), and ICAM-3 expressed on monocytes and resting lymphocytes (de Fougerolles et al., *J. Exp. Med.* 179: 619-629 (1994)). Accordingly, cell adhesion assays (e.g., using fluorescently labeled cells) can be performed between LFA-1 expressing cells and other leukocytes, endothelial cells, monocytes, and dermal fibroblasts.

Another exemplary assay for ICAM binding is as follows: ICAM-1 is purified from human tonsil, and coated on 96-well plates as described previously (Lu and Springer, (1997) *J Immunol* 159:268-278). LFA-1 expressing cells are labeled with a fluorescence dye 2', 7'-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxyethyl ester (BCECF-AM), and resuspended at about $1\cdot10^6$ /ml in L15/FBS. 50 μ l of cell suspension is mixed in ICAM-1 coated wells with an equal volume of L15/FBS in the absence or presence of a test compound (e.g., a candidate aLFA-1 binding protein). The assays can be performed in the presence and absence of an activating monoclonal antibody (CBRLFA-1/2, 10 μ g/ml).

For testing the effect of divalent cations, BCECF-AM-labeled cells are washed twice with TS buffer, pH7.5 (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5 mM EDTA, followed by two washes with TS buffer, pH7.5. Cells were then resuspended to $5\cdot10^5$ /ml in the TS buffer, pH7.5 supplemented with 1 mM MgCl₂ and other divalent cations and 2 mM EDTA. 100 μ l of the cell suspension is added to ICAM-1 coated wells. After incubation at 37°C for 30 minutes, unbound cells are washed off on a Microplate Autowasher (Bio-Tek Instruments, Winooski, Vt.). The fluorescence content of total

input cells and the bound cells in each well is quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, Me.). The number of bound cells can be expressed as a percentage of total input cells per sample well.

The following exemplary assay evaluates the effect of a test compound (e.g., an αLFA-1 binding protein) on the ability of test compound to modulate cell-cell interactions that depend on LFA-1. The assay uses lymphoma cell line EL-4 which expresses both murine LFA-1 and ICAM-1, and which exhibits LFA-1-dependent homotypic aggregation upon activation by PMA. Cells are incubated in a 96 well plate in the presence of 50 ng/ml PMA and varying amounts of the test compound. After incubation for 2 hours at 37 °C, 5% CO₂, the degree of aggregation was scored under the microscope as follows: 0 indicated that essentially no cells are clustered; 1 indicated that <10% of cells are aggregated; 2 indicated clustering of <50%; 3 indicated that up to 100% of cells were in small, loose aggregates; 4 indicated that nearly 100% of cells are in larger clusters; and 5 indicated that nearly 100% of cells are in very large, tight clusters.

Still another exemplary assay evaluates the ability of a test compound to inhibit LFA-1 function in vivo. The assay includes visualizing microcirculation in the peripheral lymph node (LN) with intravital microscopy. Briefly, a small bolus (20-50 µl) of LN cell suspensions from TGFβ mice are retrogradely injected through a femoral artery catheter and visualized in the subiliac LN by fluorescent epi-illumination from a video-triggered xenon arc stroboscope. After recording control TGFβ cell behavior in the absence of test compound, the mouse was pretreated by intra-arterial injection of the test compound (e.g., at a desired concentration) 5 minutes before T^{GFP} cell injection. Scenes can be recorded on videotape and off-line analysis was done. The rolling fraction can be calculated as percentage of the number rolling cells relative to the total number of TGFβ cells that entered a venule. The sticking (firm adhesion) fraction can be determined as the percentage of T^{GFP} cells becoming firmly adherent for >20 seconds in the number of T^{GFP} cells that rolled in a venule. Results can be semi-quantitatively scored as follows: -: 0%, .+-.: 0-5%, +: 5-20%, ++: 20-40%, +++: 40-60%, +++++: 60-80%, ++++++: 80-100%.

The vascular endothelium is a substrate with which monocytes/granulocytes can interact during adherence, diapedesis, and differentiation. An in vitro assay for monocyte/granulocyte interaction with the vessel wall consists of binding radiolabeled or

fluorescein monocyte/granulocyte preparations to cultured vascular endothelium, as described in Arnaout et al., *J. Cell Physiol.* 137:305 (1988). Mentzer et al., *J. Cell Physiol.* 125:285 (1986) describes a lymphocyte adhesion assay. A granulocyte aggregation assay can be performed as described by Arnaout et al., *New Engl. J. Med.* 306:693 (1982). Aggregation can be induced by zymosan-activated autologous serum or with chemotactic peptides, e.g. FMLP. Aggregation can then be recorded as incremental change in light transmission using a platelet aggregometer. The results can be confirmed by phase microscopy. Chemotaxis can be evaluated, e.g., as described in Dana et al., *J. Immunol.* 137:3259 (1986).

A protein (e.g., an antibody described herein) can also be evaluated in culture for ability to modulate inflammation or an inflammatory disorder. For example, , cell culture is used to monitor adhesion of leukocytes. A compound can be immobilized on a solid surface and adhesion of cells expressing an adhesion molecule can be evaluated for interaction with the surface. Cells suitable for this assay include any leukocytes, such as T cells, B cells, monocytes, eosinophils, and basophils. Exemplary leukocyte cell lines include Jurkat and U937 cells.

In one embodiment, a protein (e.g., an antibody described herein) has a statistically significant effect in an assay described herein. An assay for a protein can be compared to corresponding control assay, as appropriate, e.g., an assay lacking one or more components, e.g., lacking the test compound, a particular cell, a particular antibody, cation, etc.

Animal Models

An aLFA-1 binding protein can be evaluated in an animal model, e.g., an animal model for an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

A number of animal models for psoriasis are available. Examples include the following. Schon et al. (1997) *Nat Med.* 3:183-8 describe a mouse having a murine psoriasis-like disorder. The mouse was created by reconstituting scid/scid mice with naive CD4⁺ T cells. Other mouse models for psoriasis have also utilized

immunodeficient animals. Sugai et al. (1998) *J Dermatol Sci* 17:85-92 transplanted human psoriatic lesions onto SCID mice. Yamamoto et al. (1998) *J Dermatol Sci* 17:8-14 describe injecting staphylococcal enterotoxin B-stimulated lymphocytes subcutaneously under full-thickness psoriatic skin grafted onto severe combined immunodeficient (SCID) mice. Sundberg et al. (1997) *Pathobiology* 65(5):271-86 describe the development and progression of psoriasisiform dermatitis and systemic lesions in the flaky skin (fsn) mouse mutant. Flaky skin (fsn) mutant mice have been described as a mouse model for psoriasis accompanied by hematological abnormalities. Hong et al. (1999) *J. Immunol.* 162:7480-7491 describe additional animal models of psoriasis. US 6,410,824 describes producing an animal model by transferring naive, immunocompetent T lymphocytes into an immunodeficient animal host, along with at least one pro-inflammatory cytokine and a polyclonal activating agent. The engrafted T cells are tolerant to the major histocompatibility antigens of the host animal, but are mismatched at one or more minor histocompatibility loci. The engrafted animals develop a chronic skin disorder that includes histological features observed in human psoriasis, e.g. rete pegs, severe acanthosis and infiltration of Th1 cells into the dermis.

US 2003-0161810 provides a non-human animal model for an inflammatory disorder (including rheumatoid arthritis). The animal described therein includes human synovial fluid. US 2003-0176389 describes a dextran sodium sulfate-induced mouse model of colitis.

An aLFA-1 binding protein can be assayed for an effect on neutrophil migration. One model for neutrophil migration is murine thioglycollate induced peritonitis. Thioglycollate is injected i.p. to mice and immediately thereafter the protein to be tested is given, e.g., by i.p. or s.c. The mice are killed after 4 hours, the peritoneal cavity lavaged and the total number of neutrophils in the lavage fluid is determined.

An aLFA-1 binding protein can be assayed for an effect on ischemia/reperfusion injury. The protein can be tested, e.g., in a model of heart ischemia/reperfusion injury (Abdeslam Oubenaissa et al., *Circulation*, 94, Suppl. II, 254-258, 1996). The protein can also be tested as follows:

Mice are treated with an aLFA-1 binding protein or a control. Mice weighing 20-25 g are anaesthetized with isoflurane and the right renal vessels are clamped using

microvascular clamps for 60 min. After 60 min of ischemia, the microvascular clamps are removed. The left renal vessels (renal artery, vein and urethra) are ligated using a 4-0 surgical suture. The left (nonischemic) kidney is removed, and the abdominal cavity closed with 3-0 surgical suture. Control groups undergo the same procedures as the ischemia group, but without clamping of the right renal vessels.

Animals are sacrificed by CO₂ inhalation at 24 h, 1 week and 2 weeks following reperfusion. Blood samples are collected by cardiac puncture into a 3.0 ml VACUTAINER™ tube (Becton-Dickenson) containing 0.04 ml of a 7.5% solution of K₃ EDTA immediately after sacrifice. Plasma is separated and stored at -20°C until further analysis. Plasma creatinine and blood urea nitrogen (BUN) are analyzed. Following sacrifice, the kidney is flushed with physiological saline, immediately snap-frozen in liquid nitrogen and stored at -70°C until analysis. Myeloperoxidase activity (MPO) in the kidney can be measured according to the method of Bradley et al (*J. Invest. Dermatol.*, 78, 206-209, 1982).

An aLFA-1 binding protein can be assayed for an effect on vascularized heterotopic heart transplantation. Recipient mice are treated with an aLFA-1 binding protein or a control. Mice donor hearts are implanted onto the recipients abdominal vessels: brachiocephalic trunk to aorta and right pulmonary artery to inferior vena cava with end-to-side anastomoses using 11/0 Ethilon (Ethicon, Norderstedt, Germany) continuous sutures. Animals are closed in two layers with 6/0 Vicryl (Ethicon) and kept warm until fully recovered. Total ischaemia times are in the range of 40-50 min of which 25-35 min are at 4 °C. During anastomosis (10-15 min) the graft is kept cold.

After transplantation, graft function is monitored by daily assessment of graft beat (palpation). Rejection is considered to be complete when heart beat stops. In all experiments rejection is confirmed by histological examination of the grafts.

An exemplary assay for reperfusion injury associated with myocardial infarction in dogs is described, e.g. in Simpson et al., *J. Clin. Invest.* 81:624 (1988). Takeshima et al., *Stroke*, 23(2):247-252 (1992) describe a transient focal cerebral ischemia model in cats. Takeshima et al. used a microvascular clip to occlude the MCA and occluded CCAs by tightening previously placed ligatures. Lindsberg et al. *J. Neurosurg.* 82:269-277 (1995) describe a rabbit model of severe spinal cord ischemia (by inflating the balloon of

a catheter tip which had been introduced in the abdominal aorta). Still additional models include the reversible spinal cord model (involving a snare ligature occluding device) and an irreversible microsphere model. Clark et al., *Stroke* 22(7): 877-883 (1991).

Bowes et al., *Neurology* 45:815-819 (1995) evaluated the ability of particular antibodies to enhance the efficacy of thrombolysis in a rabbit cerebral embolism stroke model. In this model, numerous small blood clots (formed by fragmenting a clot with a tissue homogenizer) are injected into the rabbit's carotid circulation in order to achieve embolization. Neurologic function in each animal can be evaluated 18 hours following embolization on a three point scale: (1) normal activity; (2) abnormal activity; or (3) death. The amount of clot necessary to produce permanent neurologic damage in 50% of the rabbits (ED₅₀) is determined for each treatment group. Antibodies described herein can be evaluated using this or a similar model to evaluate efficacy of thrombolysis to prevent, treat, or otherwise ameliorate a stroke.

Bednar et al., *Stroke* 23(1):152 (1992) describe a rabbit model of thromboembolic stroke wherein the arterial occlusion (an autologous blood clot delivered to the anterior cerebral circulation) is not removed during the experiment. Rabbits received the binding protein (e.g., aLFA-1 binding antibody) or vehicle, 30 minutes following the thromboembolic event. Following embolization, the animals are evaluated for a total of 4 hours, including an initial 45 minutes of systemic hypotension.

An aLFA-1 binding protein can be assayed for an effect on asthma or another airway hyperresponsive disorder, e.g., using an animal model described in US 5,730,983.

In one embodiment, a protein (e.g., an antibody described herein) has a statistically significant effect in an animal model. For example, the protein has a statistically significant effect on a symptom of an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

Additional Assays

ELISA. Proteins encoded by a display library can also be screened for a binding property using an ELISA assay. For example, each protein is contacted to a microtitre plate whose bottom surface has been coated with the target, e.g., a limiting amount of the target. The plate is washed with buffer to remove non-specifically bound polypeptides.

Then the amount of the protein bound to the plate is determined by probing the plate with an antibody that can recognize the polypeptide, e.g., a tag or constant portion of the polypeptide. The antibody is linked to an enzyme such as alkaline phosphatase, which produces a colorimetric product when appropriate substrates are provided. The protein can be purified from cells or assayed in a display library format, e.g., as a fusion to a filamentous bacteriophage coat. Alternatively, cells (e.g., live or fixed) that express the target molecule, e.g., a conformationally biased LFA-1, can be plated in a microtitre plate and used to test the affinity of the peptides/antibodies present in the display library or obtained by selection from the display library.

In another version of the ELISA assay, each polypeptide of a diversity strand library is used to coat a different well of a microtitre plate. The ELISA then proceeds using a constant target molecule to query each well.

Homogeneous Binding Assays. The binding interaction of candidate protein with a target can be analyzed using a homogenous assay, i.e., after all components of the assay are added, additional fluid manipulations are not required. For example, fluorescence resonance energy transfer (FRET) can be used as a homogenous assay (see, for example, Lakowicz *et al.*, U.S. Patent No. 5,631,169; Stavrianopoulos, *et al.*, U.S. Patent No. 4,868,103). A fluorophore label on the first molecule (e.g., the molecule identified in the fraction) is selected such that its emitted fluorescent energy can be absorbed by a fluorescent label on a second molecule (e.g., the target) if the second molecule is in proximity to the first molecule. The fluorescent label on the second molecule fluoresces when it absorbs to the transferred energy. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. A binding event that is configured for monitoring by FRET can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter). By titrating the amount of the first or second binding molecule, a binding curve can be generated to estimate the equilibrium binding constant.

Another example of a homogenous assay is Alpha Screen (Packard Bioscience, Meriden CT). Alpha Screen uses two labeled beads. One bead generates singlet oxygen when excited by a laser. The other bead generates a light signal when singlet oxygen diffuses from the first bead and collides with it. The signal is only generated when the two beads are in proximity. One bead can be attached to the display library member, the other to the target. Signals are measured to determine the extent of binding.

The homogenous assays can be performed while the candidate protein is attached to the display library vehicle, e.g., a bacteriophage or using a candidate protein as free molecule.

Surface Plasmon Resonance (SPR). The binding interaction of a molecule isolated from a display library and a target can be analyzed using SPR. SPR or Biomolecular Interaction Analysis (BIA) detects biospecific interactions in real time, without labeling any of the interactants. Changes in the mass at the binding surface (indicative of a binding event) of the BIA chip result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)). The changes in the refractivity generate a detectable signal, which are measured as an indication of real-time reactions between biological molecules. Methods for using SPR are described, for example, in U.S. Patent No. 5,641,640; Raether (1988) *Surface Plasmons* Springer Verlag; Sjolander and Urbaniczky (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705 and on-line resources provide by BIACore International AB (Uppsala, Sweden).

Information from SPR can be used to provide an accurate and quantitative measure of the equilibrium dissociation constant (K_d), and kinetic parameters, including K_{on} and K_{off} , for the binding of a biomolecule to a target. Such data can be used to compare different biomolecules. For example, proteins encoded by nucleic acid selected from a library of diversity strands can be compared to identify individuals that have high affinity for the target or that have a slow K_{off} . This information can also be used to develop structure-activity relationships (SAR). For example, the kinetic and equilibrium binding parameters of matured versions of a parent protein can be compared to the parameters of the parent protein. Variant amino acids at given positions can be identified that correlate with particular binding parameters, e.g., high affinity and slow K_{off} . This

information can be combined with structural modeling (e.g., using homology modeling, energy minimization, or structure determination by crystallography or NMR). As a result, an understanding of the physical interaction between the protein and its target can be formulated and used to guide other design processes.

Protein Arrays. Polypeptides identified from the display library can be immobilized on a solid support, for example, on a bead or an array. For a protein array, each of the polypeptides is immobilized at a unique address on a support. Typically, the address is a two-dimensional address. Protein arrays are described below (see, e.g., Diagnostics).

Cellular Assays. Candidate proteins can be selected from a library by transforming the library into a host cell; the library could have been previously identified from a display library. For example, the library can include vector nucleic acid sequences that include segments that encode the polypeptides and that direct expression, e.g., such that the polypeptides are produced within the cell, secreted from the cell, or attached to the cell surface. The cells can be screened or selected for polypeptides that bind to the aLFA-1, e.g., as detected by a change in a cellular phenotype or a cell-mediated activity. For example, in the case of an antibody that binds to aLFA-1, the activity may be an *in vitro* assay for cell adhesion, cell invasion, or a lymphocyte activity.

Protein Production

Standard recombinant nucleic acid methods can be used to express an integrin binding protein. See, for example, the techniques described in Sambrook & Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory, N.Y. (2001) and Ausubel *et al.*, *Current Protocols in Molecular Biology* (Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Generally, a nucleic acid sequence encoding the binding protein is cloned into a nucleic acid expression vector. If the protein includes multiple polypeptide chains, each chain can be cloned into an expression vector, e.g., the same or different vectors, that are expressed in the same or different cells. Methods for producing antibodies are also provided below.

The expression vector for expressing the protein can include, in addition to the segment encoding the protein or fragment thereof, regulatory sequences, including for example, a promoter, operably linked to the nucleic acid(s) of interest. Vectors are typically tailored for the intended expression system. Large numbers of suitable vectors and promoters are available. The regulatory sequences can include, e.g., transcriptional regulatory sequences, splice regulatory sequences, and translational regulatory sequences.

Generally, recombinant expression vectors include origins of replication and selectable markers permitting transformation of the host cell, e.g., an antibiotic resistance gene for a bacterial cell, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. The coding sequence can be positioned in an appropriate phase relative to translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the coding sequence can include a sequence encoding a heterologous sequence, e.g., to thereby encode a fusion protein. Exemplary heterologous sequences include an identification sequence (e.g., a terminus, e.g., N- or C-terminal) imparting desired characteristics, e.g., stabilization, detection, or simplified purification of expressed recombinant product.

Prokaryotic Expression. Suitable prokaryotic hosts for expression include, e.g., *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice. Exemplary prokaryotic vectors include, for example, pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega, Madison, WI, USA), pBS, phagescript, PsiX174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia), as well as phage and phagemid vectors. Exemplary bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P, and trc. Vectors can be introduced into bacterial cells, e.g., by chemical transformation (e.g., the Hanahan protocol), electroporation, or bacteriophage infection.

If the protein is made in bacteria (or some yeast), it may be necessary to modify the protein produced therein, for example, by glycosylation of the appropriate sites, in

order to obtain the functional protein. Such covalent attachments may be accomplished using known chemical or enzymatic methods.

Yeast Expression Systems. The host cell for producing a binding protein (e.g., antibody) may also be a yeast (e.g., *Pichia*, *Hansenula*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, or *Candida*) or other fungus. In yeast, a number of vectors containing constitutive or inducible promoters may be used. Exemplary yeast promoters include the promoters of genes encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α -factor, acid phosphatase, and heat shock proteins, the PHO5 promoter, and GAL promoter, among others. A yeast vector can include a selectable marker, e.g., a drug resistance gene, or an auxotrophic marker (such as the *URA3*, *LEU2*, *HIS3*, and *TRP1* genes). It is possible to maintain yeast vectors as extrachromosomal elements in high or low copy and to integrate the vectors into yeast chromosomes (e.g., endogenous or artificial).

For a review of yeast expression systems, see, e.g., Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13 (1988); Grant *et al.*, Expression and Secretion Vectors for Yeast, in *Methods in Enzymology*, Ed. Wu & Grossman, Acad. Press, N.Y. 153:516-544 (1987); Glover, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3 (1986); Bitter, Heterologous Gene Expression in Yeast, in *Methods in Enzymology*, Eds. Berger & Kimmel, Acad. Press, N.Y. 152:673-684 (1987); Powers *et al.* (2001) *J Immunol Methods*. 251:123-35; and *The Molecular Biology of the Yeast Saccharomyces*, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and 11 (1982).

Mammalian Expression. Various mammalian cell culture systems can also be employed to express a binding protein, e.g., an antibody. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts (described by Gluzman, Cell 23:175 (1981)), the C127, 3T3, CHO, HeLa, K562, and BHK cell lines. Mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK and Jurkat cells. Mammalian expression

vectors can include an origin of replication, a suitable promoter and also any necessary ribosome-binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences.

Exemplary eukaryotic vectors include: pWLneo, pSV2cat, pOG44, PXTI, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, mouse metallothionein-I, and various art-known tissue specific promoters. In one example, DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the recombinant construct into a mammalian host cell can be effected, for example, by calcium phosphate transfection, DEAE, dextran mediated transfection, electroporation (Davis, L. *et al.*, Basic Methods in Molecular Biology (1986)), or viral infection.

In another embodiment, cells and tissues may be engineered to express an endogenous gene that encodes a binding protein described herein or a protein target (e.g., LFA-1). The method includes using homologous recombination to replace regulatory sequences of the endogenous gene with heterologous regulatory sequences, e.g., inducible regulatory elements. Such regulatory sequences may include promoters, enhancers, scaffold-attachment regions, negative regulatory elements, transcriptional initiation sites, regulatory protein binding sites or combinations of said sequences. Alternatively, sequences which affect the structure or stability of the RNA or protein produced may be replaced, removed, added, or otherwise modified by targeting, including polyadenylation signals. mRNA stability elements, splice sites, leader sequences for enhancing or modifying transport or secretion properties of the protein, or other sequences which alter or improve the function or stability of protein or RNA molecules.

Purification. Binding proteins can be purified from cells or from media surrounding cells. Cells expressing the proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Recombinant binding proteins produced in culture can be isolated by an initial extraction from cell pellets, followed by one or more salting-out, and

chromatography steps (e.g., aqueous ion exchange or size exclusion chromatography steps). In some embodiments, the protein also includes a polypeptide tag, e.g., penta- or hexa-histidine. The recombinant binding proteins can then be purified using affinity chromatography. Scopes (1994) *Protein Purification: Principles and Practice*, New York:Springer-Verlag provides a number of general methods for purifying recombinant (and non-recombinant) proteins. The methods include, e.g., ion-exchange chromatography, size-exclusion chromatography, affinity chromatography, selective precipitation, dialysis, and hydrophobic interaction chromatography. These methods can be adapted for devising a purification strategy for the integrin binding protein. For binding proteins that include an Fc domain, one type of affinity chromatography uses immobilized protein A or protein G.

Antibody Production. Some antibodies, e.g., Fabs, can be produced in bacterial cells, e.g., *E. coli* cells. For example, if the Fab is encoded by sequences in a phage display vector that includes a suppressible stop codon between the display entity and a bacteriophage protein (or fragment thereof), the vector nucleic acid can be shuffled into a bacterial cell that cannot suppress a stop codon. In this case, the Fab is not fused to the gene III protein and is secreted into the media.

Antibodies can also be produced in eukaryotic cells. In one embodiment, the antibodies (e.g., scFv's) are expressed in a yeast cell such as *Pichia* (see, e.g., Powers *et al.* (2001) *J Immunol Methods*. 251:123-35), *Hansenula*, or *Saccharomyces*.

In one embodiment, antibodies are produced in mammalian cells. Preferred mammalian host cells for expressing the clone antibodies or antigen-binding fragments thereof include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp (1982) *Mol. Biol.* 159:601-621), lymphocytic cell lines, e.g., NS0 myeloma cells and SP2 cells, COS cells, K562, and a cell from a transgenic animal, e.g., a transgenic mammal. For example, the cell is a mammary epithelial cell.

In addition to the nucleic acid sequence encoding the immunoglobulin domain, the recombinant expression vectors may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and

selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017). Exemplary selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in *dhfr*- host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

In an exemplary system for recombinant expression of an antibody (e.g., a full length antibody or an antigen-binding portion thereof), a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into *dhfr*- CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfet the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. For example, some antibodies can be isolated by affinity chromatography with a Protein A or Protein G.

For antibodies that include an Fc domain, the antibody production system may synthesize antibodies in which the Fc region is glycosylated. For example, the Fc domain of IgG molecules is glycosylated at asparagine 297 in the CH2 domain. This asparagine is the site for modification with biantennary-type oligosaccharides. This glycosylation participates in effector functions mediated by Fc_Y receptors and complement C1q (Burton and Woof (1992) *Adv. Immunol.* 51:1-84; Jefferis *et al.* (1998) *Immunol. Rev.* 163:59-76). The Fc domain can be produced in a mammalian expression system that appropriately glycosylates the residue corresponding to asparagine 297. The Fc domain can also include other eukaryotic post-translational modifications.

Antibodies can also be produced by a transgenic animal. For example, U.S 5,849,992 describes a method for expressing an antibody in the mammary gland of a transgenic mammal. A transgene is constructed that includes a milk-specific promoter and nucleic acids encoding the antibody of interest and a signal sequence for secretion. The milk produced by females of such transgenic mammals includes, secreted-therein, the antibody of interest. The antibody can be purified from the milk, or for some applications, used directly.

It is also possible to produce antibodies that bind to aLFA-1 by immunization, e.g., using an animal, e.g., with natural, human, or partially human immunoglobulin loci. Non-human antibodies can also be modified to include substitutions that insert human immunoglobulin sequences, e.g., consensus human amino acid residues at particular positions, e.g., at one or more of the following positions (preferably at least five, ten, twelve, or all): (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, and/or (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and/or 103H (according to the Kabat numbering). See, e.g., U.S. 6,407,213.

Target Protein Production. Method for producing an conformationally biased LFA-1 protein are described, e.g., in US 2002-0123614, Shimaoka, M et al. (2003) *Cell* 112,99-111 ; Shimaoka, M et al. (2002) *Annu. Rev. Biophys. Biomol. Struct.* 31,485-516; and Shimaoka, M et al. (2001) *Proc Natl Acad Sci U S A* 98:6009-6014 and Luo et al. (2003) *Proc Natl Acad Sci U S A.* 100(5):2403-8.

Biotinylation Methods. A variety of methods are available to biotinylate proteins, e.g., an immunoglobulin protein or a target protein. For example, the protein can be incubated with a 5-fold molar excess of sulfo-NHS-SS-biotin in 50 mM HEPES, pH 8.0, 100 mM NaCl overnight at 4°C. Free biotin is removed by buffer exchange into PBS, 0.01% Tween 20, e.g., using a Biomax device with a 10 kDa molecular weight cut-off membrane or by dialysis. The number of biotin molecules incorporated per mole of protein can be determined using the HABA assay as described by the manufacturer (Pierce).

Pharmaceutical Compositions

In another aspect, the invention provides compositions, e.g., pharmaceutically acceptable compositions, which include an integrin binding protein, e.g., an antibody or other protein. The integrin binding protein can be, e.g., a protein that preferentially binds to activated LFA-1, formulated together with a pharmaceutically acceptable carrier. As used herein, "pharmaceutical compositions" encompass diagnostic compositions, e.g., labeled binding proteins (e.g., for *in vivo* imaging) as well as therapeutic compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The carrier can be suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g., by injection or infusion). Depending on the route of administration, the composition may be coated in a material to protect the binding protein from the action of acids and other natural conditions that may inactivate the binding protein.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see e.g., Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

Compositions may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The form can depend on the intended mode of administration and

therapeutic application. Typical compositions are in the form of injectable or infusible solutions. One common mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). For example, the integrin binding protein is administered by intravenous infusion or injection. In another preferred embodiment, the integrin binding protein is administered by intramuscular or subcutaneous injection.

The phrases "parenteral administration" and "administered parenterally" as used herein mean modes of administration other than enteral and topical administration. Parenteral administration is usually by injection. Parenteral administration includes, *e.g.*, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Pharmaceutical compositions typically are sterile and stable under the conditions of manufacture and storage. A pharmaceutical composition can also be tested to insure it meets regulatory and industry standards for administration. For example, endotoxin levels in the preparation can be tested using the Limulus amebocyte lysate assay (*e.g.*, using the kit from Bio Whittaker lot # 7L3790, sensitivity 0.125 EU/mL) according to the USP 24/NF 19 methods. Sterility of pharmaceutical compositions can be determined using thioglycollate medium according to the USP 24/NF 19 methods. For example, the preparation is used to inoculate the thioglycollate medium and incubated at 35°C for 14 or more days. The medium is inspected periodically to detect growth of a microorganism.

The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable for delivering a high concentration of the binding protein. Sterile injectable solutions can be prepared by incorporating the binding protein in the required amount in an appropriate solvent with one or a combination of any other ingredients, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the binding protein into a sterile vehicle that contains a basic dispersion medium and any other ingredients. Sterile powders for the preparation of sterile injectable solutions can be prepared by vacuum drying and freeze-drying. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as

lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

An integrin binding protein can be administered by any appropriate method. For many applications, the route of administration is intravenous injection or infusion. For example, for therapeutic applications, the integrin binding protein can be administered by intravenous infusion. In certain embodiments, the binding protein may be prepared with a carrier that protects the protein against rapid release, such as a controlled release formulation, including implants, and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are available. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound described herein by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. The protein may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. In certain embodiments, the binding protein is administered orally, for example, with an inert diluent or an assimilable edible carrier. The protein may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's food or drink.

Pharmaceutical compositions can be administered by a medical device. For example, a pharmaceutical composition described herein can be administered with a needless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, and 4,596,556. Examples of implants and modules include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. 4,447,233, which discloses a medication infusion pump for

delivering medication at a precise infusion rate; U.S. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. 4,475,196, which discloses an osmotic drug delivery system.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of binding protein calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms can be dictated by and directly dependent on (a) the particular characteristics of the binding protein and the particular therapeutic effect to be achieved, and (b) the sensitivity of a particular individual.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody described herein is 0.01-20 mg/kg, *e.g.*, 1-10, 0.01-10, 0.03-5, 0.02-2, or 0.01-1 mg/kg. The integrin binding protein, particularly an aLFA-1 binding antibody, can be administered by intravenous infusion. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

A pharmaceutical composition may include a "therapeutically effective amount" or a "prophylactically effective amount" of an integrin binding protein, *e.g.*, aLFA-1-binding protein. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the composition may vary according to factors such

as the disease state, age, sex, and weight of the individual, and the ability of the protein to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the composition are outweighed by the therapeutically beneficial effects. A "therapeutically effective dosage" preferably inhibits a measurable parameter, e.g., parameter of inflammation by at least about 5, 10, 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit a measurable parameter, e.g., a parameter of inflammation, can be evaluated in an animal model system of inflammation. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner.

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Stabilization and Retention

In one embodiment, an integrin binding protein (e.g., an αLFA-1-binding antibody described herein or other integrin-binding protein) is physically associated with a moiety that improves its stabilization and/or retention in circulation, e.g., in blood, serum, lymph, or other tissues.

For example, an integrin binding protein can be associated with a polymer, e.g., a substantially non-antigenic polymers, such as polyalkylene oxides or polyethylene oxides. Suitable polymers will vary substantially by weight. Polymers having molecular number average weights ranging from about 200 to about 50,000, e.g., 1,000 to 15,000, 2,000 to 12,500, or 10,000 to about 30,000 can be used.

For example, an integrin binding protein can be conjugated to a water soluble polymer, e.g., hydrophilic polyvinyl polymers, e.g. polyvinylalcohol and polyvinylpyrrolidone. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided

that the water solubility of the block copolymers is maintained. Additional useful polymers include polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon.

Other compounds can also be attached to the same polymer, e.g., a cytotoxin, a label, or another targeting agent, e.g., another integrin-binding protein or an unrelated protein. Mono-activated, alkoxy-terminated polyalkylene oxides (PAO's), e.g., monomethoxy-terminated polyethylene glycols (mPEG's); C₁₋₄ alkyl-terminated polymers; and bis-activated polyethylene oxides (glycols) can be used for crosslinking. See, e.g., U.S. 5,951,974

In one embodiment, the polymer prior to cross-linking need not be, but preferably is, water soluble. Generally, after crosslinking, the product is water soluble, e.g., exhibits a water solubility of at least about 0.01 mg/ml, 0.1 mg/ml, or 1 mg/ml. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if the conjugate is intended to be administered by such routes.

In one embodiment, the polymer contains only a single group which is reactive. This feature reduces cross-linking among protein molecules. Other aspects of the reaction conditions can also be optimized to reduce cross-linking. For example, after cross-linking, the reaction products can be purified gel filtration or ion exchange chromatography to recover substantially homogenous preparations. In other embodiments, the polymer contains two or more reactive groups for the purpose of linking multiple proteins (e.g., multiple binding proteins) to the polymer backbone. Gel

filtration or ion exchange chromatography can be used to recover the desired derivative in substantially homogeneous form.

The molecular weight of the polymer can range, e.g., up to about 500,000 D, and preferably is at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. The molecular weight chosen can depend upon the effective size of the conjugate to be achieved, the nature (e.g. structure, such as linear or branched) of the polymer, and the degree of derivatization.

The covalent crosslink can be used to attach an integrin binding protein to a polymer, for example, crosslinking to the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulphydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the integrin binding protein without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, activated succinimidyl esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-nitrophenylchloroformate activated PEG.) Carboxyl groups can be derivatized by coupling PEG-amine using carbodiimide. Sulphydryl groups can be derivatized by coupling to maleimido-substituted PEG (e.g. alkoxy-PEG amine plus sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) WO 97/10847 or PEG-maleimide commercially available from Shearwater Polymers, Inc., Huntsville, Ala.). Alternatively, free amino groups on the binding protein (e.g. epsilon amino groups on lysine residues) can be thiolated with 2-imino-thiolane (Traut's reagent) and then coupled to maleimide-containing derivatives of PEG, e.g., as described in Pedley et al., *Br. J. Cancer*, 70: 1126-1130 (1994).

Functionalized PEG polymers that can be attached to an integrin binding protein are available, e.g., from Shearwater Polymers, Inc. (Huntsville, Ala.). Exemplary PEG derivatives include, e.g., amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of

carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxy carbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives may vary depending on the integrin binding protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

The conjugates of an integrin binding protein and a polymer can be separated from the unreacted starting materials, e.g., by gel filtration or ion exchange chromatography, e.g., HPLC. Heterologous species of the conjugates are purified from one another in the same fashion. Resolution of different species (e.g. containing one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids. See, e.g., WO 96/34015.

Treatments

Proteins that bind to an activated integrin, e.g., aLFA-1, have therapeutic and prophylactic utilities. For example, these binding proteins can be administered to a subject to treat or prevent a disorder, particularly inflammation, an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

A binding protein that preferentially binds to aLFA-1 can be used to prevent leukocytes with aLFA-1 from interacting with a cognate ligand of LFA-1. The aLFA-1 binding protein can reduce the ability of the leukocyte from interacting with other cells or the extracellular matrix. For example, the binding protein can reduce the ability of the leukocyte to interact with an endothelial cell.

Because the binding protein preferentially binds to aLFA-1, a lower concentration of the binding protein may be effective to inhibiting such interactions, relative to the

concentration required to achieve an equivalent effect using a binding protein that does not have a preference for αLFA-1 relative to non-activated conformations of LFA-1.

The integrin binding protein can be administered in an amount effective to ameliorate at least one symptom of inflammation, e.g., cause a statistically significant change in a parameter of inflammation. Exemplary parameters include: local temperature, core temperature, swelling (e.g., as measured), redness, local or systemic white blood cell count, presence or absence of neutrophils, cytokine levels, and elastase activity. For quantitative parameters, the degree of change can be, e.g., at least 10, 20, 30, 50, or 80%.

The integrin binding protein can be administered in an amount effective to reduce inflammation. Medical professionals can examine the subject to evaluate extent of inflammation.

The integrin binding protein can be administered in an amount effective to reduce leukocyte activity. Exemplary leukocyte activities include migration and homing to sites on inflammation, adherence to the endothelium. In one embodiment, the binding protein is administered locally, e.g., to reduce local concentration of the leukocyte.

The integrin binding protein can be administered as part of a regimen, e.g., of multiple bolus doses. In one embodiment, the doses can also include the same (or within 20, or 10% of the same) amount of the protein. In another embodiment, the initial dose is greater or less than one or more subsequent doses, e.g., at least 10, 20, 50, 60, 70, or 80% greater or less than.

The dose can be selected or titrated, e.g., to achieve a detectable serum concentration whose mean trough concentration is less than 9, 7, 6, 5, 4, 3, 2, 1, 0.3, 0.1, 0.03, or 0.01 μg/ml of the integrin binding protein.

As used herein, the term “treat” or “treatment” is the administration of an integrin binding protein to a subject. The protein can be administered alone or in combination with a second agent to a subject, an isolated tissue, or a cell. The protein can be administered to prevent or ameliorate the disorder, one or more symptoms of the disorder or a predisposition toward the disorder. Treating a cell includes modulation of an activity (e.g., function or viability) of the cell. Exemplary functions of leukocytes that can be modulated include binding, migration, adhesion, and a T cell function. The modulation

can reduce the ability of a cell to mediate a disorder, e.g., inflammation, an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder. Another example is an activity that, directly or indirectly, reduces inflammation or an indicator of inflammation. For example, the reduction can reduce a lymphocyte activity.

An integrin binding protein can also be used to prevent a disorder, e.g., inflammation, inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder. A prophylactic treatment can be effective, upon single- or multiple-dose administration to the subject, in preventing or delaying the occurrence of the onset or recurrence of a disorder, e.g., inflammation, an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

As used herein, the term "subject" includes human and non-human animals. Human subjects include a human patient having or suspected of having a disorder inflammation, inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

The term "non-human animals" includes all vertebrates, e.g., non-mammals (such as chickens, amphibians, reptiles) and mammals, such as non-human primates, sheep, dog, cow, pig, etc. For example, the subject can be a non-human mammal that has cells that can express LFA-1 or an LFA-1-like antigen with which an antibody described herein cross-reacts. Moreover, an aLFA-1-binding protein can be administered to a non-human mammal expressing LFA-1 or an LFA-1-like antigen with which the binding protein interacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of the binding protein (e.g., testing of dosages and time courses of administration).

The aLFA-1-binding proteins can selectively inhibit, inactivate, or kill cells that have activated LFA-1, e.g., to reduce inflammation, a leukocyte population, or leukocyte activity. For example, the aLFA-1binding protein can be conjugated to an agent, e.g., a cytotoxic agent such as a toxin, radioisotope, or short-range, high-energy α -emitters.

The aLFA-1 binding proteins can be used directly *in vivo* to inhibit, inactivate, or kill cells that presentactivated LFA-1 via natural complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). In one embodiment, the protein includes a complement binding effector domain, such as an Fc portion (e.g., functional portion) from IgG1, -2, or -3 or corresponding portions of IgM which bind complement. Also encompassed by the invention is a method of killing or ablating which involves using the aLFA-1 binding proteins for prophylaxis. For example, these materials can be used to prevent or delay development or progression of inflammatory disease.

An aLFA-1 binding protein can be administered in combination with one or more of the existing modalities for treating a disorder described herein. "Combination" refers to the overlapping administration. For example, a subject may be receiving an aLFA-1 binding protein and another therapy, e.g., another therapeutic agent, but the subject may not be administered both therapies at the same instant. For example, the subject may receive a first injection with the aLFA-1 binding protein, and then receive a separate injection with another therapeutic agent. In another example, the aLFA-1 binding protein and the other agent are administered together in a single injection.

Regarding exemplary combinations, the aLFA-1 binding protein can be used in combination with cyclosporins, rapamycins or ascomycins, or their immunosuppressive analogs, e.g. cyclosporin A, cyclosporin G, FK-506, rapamycin, 40-O-(2-hydroxyethyl)-rapamycin etc.; corticosteroids; cyclophosphamide; azathioprene; methotrexate; brequinar; FTY 720; leflunomide; mnizoribine; mycophenolic acid; mycophenolate mofetil; 15-deoxyspergualine; immunosuppressive monoclonal antibodies, e.g., monoclonal antibodies to leukocyte receptors, e.g., MHC, CD2, CD3, CD4, CD7, CD25, CD28, B7, CD45, or CD58 or their ligands; or other immunomodulatory compounds, e.g. CTLA4Ig, or other adhesion molecule inhibitors, e.g. mAbs or low molecular weight inhibitors including selectin antagonists and VLA-4 antagonists. These combination therapies can be part of an immunomodulating regimens or a regimen for the treatment or prevention of allo- or xenograft acute or chronic rejection, an inflammatory disorder, or an autoimmune disorders.

Inflammatory disorders

Exemplary inflammatory disorders include: acute and chronic immune and autoimmune pathologies (such as, but not limited to, rheumatoid arthritis (RA), juvenile chronic arthritis (JCA)), dermatological diseases (such as psoriasis and contact dermatitis), graft versus host disease (GVHD), scleroderma, diabetes mellitus, allergy; asthma, acute or chronic immune disease associated with an allogenic transplantation, such as, but not limited to, renal transplantation, cardiac transplantation, bone marrow transplantation, liver transplantation, pancreatic transplantation, small intestine transplantation, lung transplantation and skin transplantation; chronic inflammatory pathologies such as, but not limited to, sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology or disease; vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, Kawasaki's pathology and vasculitis syndromes, such as, but not limited to, polyarteritis nodosa, Wegener's granulomatosis, Henoch-Schonlein purpura, giant cell arthritis and microscopic vasculitis of the kidneys; chronic active hepatitis; Sjogren's syndrome; psoriatic arthritis; ophthalmic inflammatory diseases; enteropathic arthritis; reactive arthritis and arthritis associated with inflammatory bowel disease; infection diseases (such as septic shock, traumatic shock); and uveitis.

An aLFA-1-binding protein can be used to treat or prevent one of the foregoing diseases or disorders. For example, the protein can be administered (locally or systemically) in an amount effective to ameliorate at least one symptom of the respective disease or disorder. The protein may also ameliorate inflammation, e.g., a parameter of inflammation, e.g., such as local temperature, swelling (e.g., as measured), redness, local or systemic white blood cell count, presence or absence of neutrophils, cytokine levels, elastase activity, and so forth. It is possible to evaluate a subject, e.g., prior, during, or after administration of the protein, for one or more of parameters of inflammation, e.g., an aforementioned parameter.

IBD. Inflammatory bowel diseases (IBD) include generally chronic, relapsing intestinal inflammation. IBD refers to two distinct disorders, Crohn's disease and ulcerative colitis (UC). The clinical symptoms of IBD include intermittent rectal bleeding, crampy abdominal pain, weight loss and diarrhea. A clinical index can also be

used to monitor IBD such as the Clinical Activity Index for Ulcerative Colitis. See also, e.g., Walmsley et al. *Gut*. 1998 Jul;43(1):29-32 and Jowett et al. (2003) *Scand J Gastroenterol.* 38(2):164-71. An integrin binding protein (e.g., an aLFA-1 binding antibody described herein) can be used to ameliorate at least one symptom of IBD or to ameliorate a clinical index of IBD.

Psoriasis. Psoriasis is a chronic skin disease, characterized by scaling and inflammation. Psoriasis affects 1.5 to 2 percent of the United States population, or almost 5 million people. When psoriasis develops, typically patches of skin thicken, redden, and become covered with silvery scales, referred to as plaques. Psoriasis most often occurs on the elbows, knees, scalp, lower back, face, palms, and soles of the feet. The disease also may affect the fingernails, toenails, and the soft tissues inside the mouth and genitalia. About 10 percent of people with psoriasis have joint inflammation that produces symptoms of arthritis.

The chronic skin inflammation of psoriasis is associated with hyperplastic epidermal keratinocytes and infiltrating mononuclear cells, including CD4+ memory T cells, neutrophils and macrophages.

Patients can be evaluated using a static Physician Global Assessment (sPGA), and receive a category score ranging from six categories between clear and very severe. The score is based on plaque, scaling, and erythema.

An integrin binding protein (e.g., an aLFA-1 binding antibody described herein) can be used to ameliorate at least one symptom of psoriasis or to ameliorate a clinical index of psoriasis (e.g., sPGA index). The protein can be administered locally or systemically.

Asthma

Asthma is a heterogeneous family of diseases. It is characterized by a hyper-responsiveness of the tracheobronchi to stimuli (McFadden, E. R. et al., In: Harrison's Principles of Internal Medicine, 10th Ed., Petersdorf, R. G. et al., Eds., McGraw-Hill, NY (1983), pages 1512-1519); Kay, A. B., Allergy and Inflammation, Academic Press, NY (1987); which references are incorporated herein by reference). Clinically, asthma is

manifested by the extensive narrowing of the tracheobronchi, by thick tenacious secretions, by paroxysms of dyspnea, cough, and wheezing. Although the relative contribution of each of these conditions is unknown, the net result is an increase in airway resistance, hyperinflation of the lungs and thorax, abnormal distribution of ventilation and pulmonary blood flow. The disease is manifested in episodic periods of acute symptoms interspersed between symptom-free periods. The acute episodes result in hypoxia, and can be fatal. Approximately 3% of the general world population suffers from the disease.

As used herein, "asthma" refers to either allergic or idiosyncratic asthma. Allergic asthma is usually associated with a heritable allergic disease, such as rhinitis, urticaria, eczema, etc. The condition is characterized by positive wheal-and-flare reactions to intradermal injections of airborne antigens (such as pollen, environmental or occupational pollutants, etc.), and increased serum levels of IgE. The development of allergic asthma appears to be causally related to the presence of IgE antibodies in many patients. Asthma patients who do not exhibit the above-described characteristics are considered to have idiosyncratic asthma.

An integrin binding protein (e.g., an αLFA-1 binding antibody described herein) can be used to ameliorate at least one symptom of asthma or to ameliorate a clinical index of asthma (e.g., airway responsiveness). The protein can be administered locally (e.g., by inhalation) or systemically (e.g., by injection).

Ischemia/Stroke and other Cardiovascular disorders

The binding proteins described herein can also be used to treat or prevent cardiovascular disorders in which LFA-1 is a factor. Such disorders include, e.g., ischemia/reperfusion injury, e.g., leukocyte-mediated reperfusion damage (e.g., post thrombolytic therapy), myocardial infarction, stroke, gut ischemia, and renal failure or hemorrhage shock.

An integrin binding protein (e.g., an αLFA-1 binding antibody described herein) can be administered to a subject who is at risk for one of the above disorders, e.g., at risk for a stroke, or to a subject who had a stroke or other cardiovascular dysfunction. For example, the binding protein can be administered before, during, or immediately after, or

any other time after such a stroke or other cardiovascular dysfunction, e.g., within 2, 4, 6, 12, 24, or 48 hours. In one embodiment, the binding protein is administered to reach a desired circulating concentration for at least 1, 2, 4, 5, 7, or 10 days.

An integrin binding protein (e.g., aLFA-1 binding protein, e.g., an aLFA-1 binding antibody described herein) can be used to treat a focal ischemic stroke, e.g., a thromboembolic stroke, or a cerebral ischemic stroke. "Focal ischemic stroke" is defined as damage to the brain caused by interruption of the blood supply to a region, generally caused by obstruction of any one or more of the "main cerebral arteries" (e.g. middle cerebral artery, anterior cerebral artery, posterior cerebral artery, internal carotid artery, vertebral artery or basilar artery). The "arterial obstruction" is generally a single embolus or thrombus. A cerebral embolism stroke can result from the obstruction of secondary arteries or arterioles, e.g., as in the model of Bowes et al., Neurology 45:815-819 (1995) in which a plurality of clot particles occlude secondary arteries or arterioles.

The aLFA-1 binding protein can be administered to increase cerebral blood flow can be increased and/or reduce infarct size in a subject having suffered the stroke. The administering can be provided, e.g., prior to removal of the arterial obstruction. For example, the obstruction is not removed until a therapeutic benefit, e.g., such as increased cerebral blood flow is detected. The method can be performed without administering a thrombolytic agent.

The aLFA-1 binding protein can be administered to a patient as soon as possible once the condition of acute ischemic stroke has been diagnosed, e.g., as suggested by focal deficit on neurologic examination. Neurologic examination and, optionally, neuroimaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) (including diffusion weighted imaging (DWI) and perfusion imaging (PI)); vascular imaging (e.g., duplex scanning and transcranial Doppler ultrasound and laser Doppler); angiography (e.g., computerized digital subtraction angiography (DSA) and MR angiography) as well as other invasive or non-invasive techniques can be used to diagnose acute ischemic stroke.

The aLFA-1 binding protein can be administered at least once or continuously at any time from immediately following to about 24 hours after the onset of stroke. In certain embodiments, the aLFA-1 binding protein is first administered to the patient at a

time between about 15 minutes (or 30 minutes or 45 minutes) to about 5 hours (or 12 hours or 24 hours) from the onset of stroke. For example, the aLFA-1 binding protein may be first administered by bolus dosage as soon as stroke is diagnosed, followed by a subsequent bolus dosage of the antagonist (e.g. 5-24 hours after the initial bolus dosage). In another example the protein is administered continuously.

Cancer

An integrin binding protein (e.g., an aLFA-1 binding antibody described herein) can be used to treat a proliferative disorder of T-cells, e.g., a T cell leukemia or lymphoma. In one embodiment, the disorder is acute promyelocytic leukemia. Other exemplary disorders that can be treated include myeloid disorders, such as acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) *Crit Rev. in Oncol./Hematol.* 11:267-97). Lymphoid malignancies that may be treated include, e.g., acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, e.g., non-Hodgkin's lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

Diagnostic Uses

Binding proteins that bind to an activated integrin (e.g., aLFA-1), can also be used for diagnostics *in vitro* and *in vivo*. In one aspect, the invention provides a diagnostic method for detecting the presence of an aLFA-1 *in vitro* or *in vivo* (e.g., *in vivo* imaging in a subject).

In one embodiment, the integrin binding protein is used to evaluate a sample *in vitro* (e.g., a biological sample). The method includes: (i) contacting a sample with aLFA-1-binding protein; and (ii) detecting formation of a complex between the aLFA-1-binding protein and the sample. The method can also include contacting a reference sample (e.g., a control sample) with the binding protein, and determining the extent of formation of the

complex between the binding protein and the sample, relative to the same for the reference sample. A change, e.g., a statistically significant change, in the formation of the complex in the sample or subject relative to the control sample or subject can be indicative of the presence of aLFA-1 in the sample. Samples can be obtained by surgical or non-surgical methods.

Another method includes: (i) administering the aLFA-1-binding protein to a subject; and (ii) detecting formation of a complex between the aLFA-1-binding protein, and the subject. The detecting can include determining location or time of formation of the complex. In one embodiment, the subject has, is suspected of having, or is at risk for a disorder described herein, e.g., an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder.

The aLFA-1-binding protein can be directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials.

Complex formation between the aLFA-1-binding protein and aLFA-1 can be detected by measuring or visualizing either the binding protein bound to the aLFA-1 or unbound binding protein. Conventional detection assays can be used, e.g., an enzyme-linked immunosorbent assays (ELISA), a radioimmunoassay (RIA) or tissue immunohistochemistry. Further to labeling the aLFA-1-binding protein, the presence of aLFA-1 can be assayed in a sample by a competition immunoassay utilizing standards labeled with a detectable substance and an unlabeled aLFA-1-binding protein. In one example of this assay, the biological sample, the labeled standards and the aLFA-1 binding agent are combined and the amount of labeled standard bound to the unlabeled binding protein is determined. The amount of aLFA-1 in the sample is inversely proportional to the amount of labeled standard bound to the aLFA-1 binding agent.

Fluorophore and chromophore labeled binding proteins can be prepared. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties should be selected to have substantial absorption at wavelengths above 310 nm and preferably above 400 nm. A variety of suitable fluorescers and chromophores are described by Stryer (1968) *Science*, 162:526 and Brand, L. et al.

(1972) *Annual Review of Biochemistry*, 41:843-868. The binding proteins can be labeled with fluorescent chromophore groups by conventional procedures such as those disclosed in U.S. Patent Nos. 3,940,475, 4,289,747, and 4,376,110. One group of fluorescers having a number of the desirable properties described above is the xanthene dyes, which include the fluoresceins and rhodamines. Another group of fluorescent compounds are the naphthylamines. Once labeled with a fluorophore or chromophore, the binding protein can be used to detect the presence or localization of the aLFA-1 in a sample, e.g., using fluorescent microscopy (such as confocal or deconvolution microscopy).

Histological Analysis. Immunohistochemistry can be performed using the binding proteins described herein. For example, in the case of an antibody, the antibody can be synthesized with a label (such as a purification or epitope tag), or can be detectably labeled, e.g., by conjugating a label or label-binding group. For example, a chelator can be attached to the antibody. The antibody is then contacted to a histological preparation, e.g., a fixed section of tissue that is on a microscope slide. After an incubation for binding, the preparation is washed to remove unbound antibody. The preparation is then analyzed, e.g., using microscopy, to identify if the antibody bound to the preparation.

The antibody (or other polypeptide or peptide) can be unlabeled at the time of binding. After binding and washing, the antibody is labeled in order to render it detectable.

Protein Arrays. The aLFA-1-binding protein can also be immobilized on a protein array. The protein array can be used as a diagnostic tool, e.g., to screen medical samples (such as isolated cells, blood, sera, biopsies, and the like). Of course, the protein array can also include other binding proteins, e.g., that bind to aLFA-1 or to other target molecules, such as hyaluronic acid.

Methods of producing protein arrays are described, e.g., in De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res.* 28, e3, I-VII; MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 01/40803 and WO 99/51773A1. Proteins for the array can be spotted at high speed, e.g., using commercially available robotic apparatus, e.g., from Genetic MicroSystems or BioRobotics. The array substrate can be, for example,

nitrocellulose, plastic, glass, e.g., surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

For example, the array can be an array of antibodies, e.g., as described in De Wildt, *supra*. Cells that produce the binding proteins can be grown on a filter in an arrayed format. Protein production is induced, and the expressed proteins are immobilized to the filter at the location of the cell.

A protein array can be contacted with a labeled target to determine the extent of binding of the target to each immobilized polypeptide from the diversity strand library. If the target is unlabeled, a sandwich method can be used, e.g., using a labeled probe, to detect binding of the unlabeled target.

Information about the extent of binding at each address of the array can be stored as a profile, e.g., in a computer database. The protein array can be produced in replicates and used to compare binding profiles, e.g., of a target and a non-target. Thus, protein arrays can be used to identify individual members of the diversity strand library that have desired binding properties with respect to one or more molecules.

An αLFA-1-binding protein described herein can also be used to detecting binding of an αLFA-1 to an insoluble support. For example, a sample can be immobilized on array, and αLFA-1 can be detected on the array using the αLFA-1-binding protein.

FACS. (Fluorescent Activated Cell Sorting). The αLFA-1-binding protein can be used to label cells, e.g., cells in a sample (e.g., a patient sample). For example, the protein can be used to detect activated integrins on cells (e.g., activated LFA-1). The binding protein is typically physically associated with (or attachable to) a fluorescent compound. The cells can then be sorted using fluorescent activated cell sorted (e.g., using a sorter available from Becton Dickinson Immunocytometry Systems, San Jose CA; see also U.S. 5,627,037; 5,030,002; and 5,137,809). As cells pass through the sorter, a laser beam excites the fluorescent compound while a detector counts cells that pass through and determines whether a fluorescent compound is attached to the cell by detecting fluorescence. The amount of label bound to each cell can be quantified and analyzed to characterize the sample.

The sorter can also deflect the cell and separate cells bound by the binding protein from those cells not bound by the binding protein. The separated cells can be cultured and/or characterized.

In vivo Imaging. Integrin binding proteins can be used to detect the presence of cells that include an activated integrin, e.g., cells presenting aLFA-1, *in vivo*. The method includes (i) administering to a subject (e.g., a patient having an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder) an aLFA-1-binding antibody, conjugated to a detectable marker; (ii) exposing the subject to a means for detecting the detectable marker. For example, the subject is imaged, e.g., by NMR or other tomographic means.

Examples of labels useful for diagnostic imaging include radiolabels such as ¹³¹I, ¹¹¹In, ¹²³I, ^{99m}Tc, ³²P, ¹²⁵I, ³H, ¹⁴C, and ¹⁸⁸Rh, fluorescent labels such as fluorescein and rhodamine, nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed. The binding protein can be labeled with such reagents using known techniques. For example, see Wensel and Meares (1983) *Radioimmunoimaging and Radioimmunotherapy*, Elsevier, New York for techniques relating to the radiolabeling of antibodies and D. Colcher et al. (1986) *Meth. Enzymol.* 121: 802-816.

A radiolabeled binding protein can also be used for *in vitro* diagnostic tests. The specific activity of a isotopically-labeled binding protein depends upon the half-life, the isotopic purity of the radioactive label, and how the label is incorporated into the antibody.

Procedures for labeling polypeptides with the radioactive isotopes (such as ¹⁴C, ³H, ³⁵S, ¹²⁵I, ³²P, ¹³¹I) are generally known. For example, tritium labeling procedures are described in U.S. Patent No. 4,302,438. Iodinating, tritium labeling, and ³⁵S labeling procedures, e.g., as adapted for murine monoclonal antibodies, are described, e.g., by Goding, J.W. (*Monoclonal antibodies : principles and practice : production and application of monoclonal antibodies in cell biology, biochemistry, and immunology* 2nd ed. London ; Orlando : Academic Press, 1986. pp 124-126) and the references cited

therein. Other procedures for iodinating polypeptides, such as antibodies, are described by Hunter and Greenwood (1962) *Nature* 144:945, David et al. (1974) *Biochemistry* 13:1014-1021, and U.S. Patent Nos. 3,867,517 and 4,376,110. Exemplary radio-isotopes that are useful for imaging include ^{123}I , ^{131}I , ^{111}In , and ^{99m}Tc . Procedures for iodinating antibodies are described by Greenwood, F. et al. (1963) *Biochem. J.* 89:114-123; Marchalonis, J. (1969) *Biochem. J.* 113:299-305; and Morrison, M. et al. (1971) *Immunochemistry* 289-297. Procedures for ^{99m}Tc -labeling are described by Rhodes, B. et al. in Burchiel, S. et al. (eds.), *Tumor Imaging: The Radioimmunochemical Detection of Cancer*, New York: Masson 111-123 (1982) and the references cited therein. Procedures suitable for ^{111}In -labeling antibodies are described by Hnatowich, D.J. et al. (1983) *J. Immunol. Methods*, 65:147-157, Hnatowich, D. et al. (1984) *J. Applied Radiation*, 35:554-557, and Buckley, R. G. et al. (1984) *F.E.B.S.* 166:202-204.

In the case of a radiolabeled binding protein, the binding protein is administered to the patient, is localized to cells with which the binding protein reacts, and is detected or "imaged" *in vivo* using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. See e.g., A.R. Bradwell et al., "Developments in Antibody Imaging", *Monoclonal Antibodies for Cancer Detection and Therapy*, R.W. Baldwin et al., (eds.), pp 65-85 (Academic Press 1985). Alternatively, a positron emission transaxial tomography scanner, such as designated Pet VI located at Brookhaven National Laboratory, can be used where the radiolabel emits positrons (e.g., ^{11}C , ^{18}F , ^{15}O , and ^{13}N).

MRI Contrast Agents. Magnetic Resonance Imaging (MRI) uses NMR to visualize internal features of living subject, and is useful for prognosis, diagnosis, treatment, and surgery. MRI can be used without radioactive tracer compounds for obvious benefit. Some MRI techniques are summarized in EP-A-0 502 814. Generally, the differences related to relaxation time constants T1 and T2 of water protons in different environments are used to generate an image. However, these differences can be insufficient to provide sharp high resolution images.

The differences in these relaxation time constants can be enhanced by contrast agents. Examples of such contrast agents include a number of magnetic agents paramagnetic agents (which primarily alter T1) and ferromagnetic or superparamagnetic

(which primarily alter T2 response). Chelates (e.g., EDTA, DTPA and NTA chelates) can be used to attach (and reduce toxicity) of some paramagnetic substances (e.g., Fe^{+3} , Mn^{+2} , Gd^{+3}). Other agents can be in the form of particles, e.g., less than 10 μm to about 10 nM in diameter). Particles can have ferromagnetic, antiferromagnetic or superparamagnetic properties. Particles can include, e.g., magnetite (Fe_3O_4), $\gamma\text{-Fe}_2\text{O}_3$, ferrites, and other magnetic mineral compounds of transition elements. Magnetic particles may include: one or more magnetic crystals with and without nonmagnetic material. The nonmagnetic material can include synthetic or natural polymers (such as sepharose, dextran, dextrin, starch and the like).

The aLFA-1-binding proteins can also be labeled with an indicating group containing of the NMR-active ^{19}F atom, or a plurality of such atoms inasmuch as (i) substantially all of naturally abundant fluorine atoms are the ^{19}F isotope and, thus, substantially all fluorine-containing compounds are NMR-active; (ii) many chemically active polyfluorinated compounds such as trifluoracetic anhydride are commercially available at relatively low cost, and (iii) many fluorinated compounds have been found medically acceptable for use in humans such as the perfluorinated polyethers utilized to carry oxygen as hemoglobin replacements. After permitting such time for incubation, a whole body MRI is carried out using an apparatus such as one of those described by Pykett (1982) *Scientific American*, 246:78-88 to locate and image activated leukocytes.

Information obtained from evaluating an aLFA-1-binding protein, e.g., a binding protein described herein, can be recorded on machine-compatible media, e.g., computer readable or computer accessible media. The information can be stored as a computer representation, e.g., in a database (e.g., in the case of imaging using a binding protein, a database of images for one or a plurality of subjects). The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

Kits

Also within the scope of the invention are kits that include a composition described herein, e.g., a composition that contains an aLFA-1-binding protein. In one

embodiment, the kit includes (a) a composition that includes the aLFA-1-binding protein, and, optionally, (b) informational material. The informational material can be descriptive, instructional, marketing or other material that relates to the methods described herein and/or the use of the compound for the methods described herein, e.g., a treatment, prophylactic, or diagnostic use. For example, the informational material describes methods for administering the composition to treat a disorder, e.g., an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or other LFA-1 mediated disorder.

In one embodiment, the informational material can include instructions to administer the compound in a suitable manner, e.g., in a suitable dose, dosage form, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). In another embodiment, the informational material can include instructions for identifying a suitable subject, e.g., a human, e.g., a human having, or at risk for an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder. The informational material can include information about production of the compound, molecular weight of the compound, concentration, date of expiration, batch or production site information, and so forth. The informational material of the kits is not limited in its form. Information about the compound can include structural information, e.g., amino acid sequence, tradename, FDA approved name, antibody isotype, and so forth. In many cases, the informational material, e.g., instructions, is provided in printed matter, e.g., a printed text, drawing, and/or photograph, e.g., a label or printed sheet. However, the informational material can also be provided in other formats, such as computer readable material, video recording, or audio recording. In another embodiment, the informational material of the kit is a link or contact information, e.g., a physical address, email address, hyperlink, website, or telephone number, where a user of the kit can obtain substantive information about the compound and/or its use in the methods described herein. The informational material can also be provided in any combination of formats.

In addition to the composition that includes the aLFA-1-binding protein, the composition itself can include other ingredients, such as a solvent or buffer, a stabilizer or a preservative, and/or a second agent for treating a condition or disorder described herein,

e.g., an inflammatory disorder, a disorder characterized by excessive LFA-1 activity, or a LFA-1 mediated disorder. Alternatively, such other ingredients can be included in the kit, but in different compositions or containers than the composition that includes the aLFA-1-binding protein. In such embodiments, the kit can include instructions for admixing the compound and the other ingredients, or for using the compound together with the other ingredients.

The composition that includes the aLFA-1-binding protein can be provided in any form, e.g., liquid, dried or lyophilized form. The composition can be substantially pure and/or sterile. When the composition that includes the aLFA-1-binding protein is provided in a liquid solution, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being preferred. When the composition that includes the aLFA-1-binding protein is provided as a dried form, reconstitution generally is by the addition of a suitable solvent. The solvent, e.g., sterile water or buffer, can optionally be provided in the kit.

The kit can include one or more containers for the composition that includes the aLFA-1-binding protein. In some embodiments, the kit contains separate containers, dividers or compartments for the composition and informational material. For example, the composition can be contained in a bottle, vial, or syringe, and the informational material can be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the composition is contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label. In some embodiments, the kit includes a plurality (e.g., a pack) of individual containers, each containing one or more unit dosage forms (e.g., a dosage form described herein) of the aLFA-1-binding protein. For example, the kit includes a plurality of syringes, ampules, foil packets, or blister packs, each containing a single unit dose of the compound. The containers of the kits can be air tight, waterproof (e.g., impermeable to changes in moisture or evaporation), and/or light-tight.

Kits can be provided that include an aLFA-1-binding antibody and instructions for diagnostic, e.g., the use of the aLFA-1-binding protein (e.g., antibody or antigen-binding fragment thereof, or other polypeptide or peptide) to detect aLFA-1, *in vitro*, e.g.,

in a sample, e.g., a biopsy or cells from a patient having a disorder described herein, or *in vivo*, e.g., by imaging a subject. The kit can further contain a least one additional reagent, such as a label or additional diagnostic agent. For *in vivo* use the ligand can be formulated as a pharmaceutical composition.

The following invention is further illustrated by the following examples, which should not be construed as limiting.

EXAMPLES

The D2-57 Fab was isolated by depleting a Fab phage display library on the low affinity wild type purified I domain protein followed by positive selection on high affinity locked open form I domain LFA-1 protein. D2-57 Fab binds to the high affinity locked open state of purified I domain protein in the presence, but not absence of magnesium. It does not bind significantly to the low affinity locked closed state of purified I domain protein in either the presence or absence of magnesium.

In the presence of magnesium, D2-57 Fab binds to cells expressing whole LFA-1 when the α subunit contains an I domain in the high affinity locked open state, but not when magnesium is absent. It also binds in the presence of magnesium to activated wild-type LFA-1 protein expressed on cells, but not to cells expressing whole LFA-1 when the α subunit contains an I domain in the low affinity locked closed state.

The reformatted full IgG1, when tested with cells, has the same binding specificities as the Fab form.

Phage that display the P1-G10 in a Fab format bind to the high affinity locked open form I domain LFA-1 protein in the presence or absence of magnesium, but do not bind to the low affinity locked closed state.

The C1-54 Fab binds to both the high affinity locked open form I domain LFA-1 protein and the low affinity locked closed state, but preferentially binds to the open form in the presence of magnesium.

The following is a comparison of the variable region of exemplary antibodies:

VARIABLE REGION - LIGHT CHAINS

	FR1-L	CDR1-L	FR2-L	CDR2-L
D2-57	QDIQMTOQPSSLSASVGDRVTITC	RASQSISGYLN	WYQQKTKAPKALIY	AASSLQS
C1-54	QDIQMTOQPATLSVSPGERVTLSC	TASQVSDSNLIA	WYQQKPGQAPRLLVY	GASTRAT
P1-G10	QSV.LTQ.PPSVSVSPGQTASVTC	SGDALGQKYAS	WYQQKPGQSPVLVIF	QDSKRPS

	FR3-L	CDR3-L	FR4-L
D2-57	GVPSRFGSGSGTDFTLTISSLQLEDFATYYC	QQSYSTP..S	FQQGTKVEIKRT
C1-54	GVPARFSGSGSGTAFTLTIDSLQSEDFAVYYC	QQYNKWPPYS	FQQGTKLEIKRT
P1-G10	GIPERFSGNSGNATLTISGTQAVDEADYYC	QAWDTT.AYV	FGTGTVTTLV

VARIABLE REGION - HEAVY CHAINS

	FR1-H	CDR1-H	FR2-H
D2-57	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RVVMW	WVRQAPGKGLEWVS
C1-54	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	HYGMS	WVRQAPGKGLEWVS
P1-G10	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	HYSMQ	WVRQAPGKGLEWVS

	CDR2-H
D2-57	YIWPSSGGNTYYADSVKG
C1-54	VISPSSGGRTLYADSVKG
P1-G10	YIGSSGGNTYYADSVKG

	FR3-H	CDR3-H	FR4-H
D2-57	RFTISRDNSKNLTYLQMNSLRAEDTAVYYCAS	SYDFWSNAFDI	WGQGTMVTVSS
C1-54	RFTISRDNSKNLTYLQMNSLRAEDTAVYYCAC	HYSY...AMDV	WGQGTTVTVSS
P1-G10	RFTISRDNSKNLTYLQMNSLRAEDTAVYYCAR	G.TYNTSPFDY	WGQGTLTVTVSS

VARIABLE REGION -LIGHT CHAINS (Nucleic Acid)

	FR1-L
D2-57	CAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGGAGACAGAGTCAC
C1-54	CAAGACATCCAGATGACCCAGTCTCCAGGCCACCCCTGTCTGTGTCAGGGAAAGAGTCAC
P1-G10	CAGAGCGCTTGA.....CTCAGGCCACCCCTCAGTGTCAGGTCTCCCCAGGACAGACAGCCAG

	CDR1-L	
D2-57	CATCACTTGC CGGGCAAGTCAGAGCATTGGCAGCTACTTAAAC	TGGTATCAGCAGAAAAAC
C1-54	CCTCTCCTGC ACGGCCAGTCAGAGTGTGACAGCAACTTAGCC	TGGTATCAGCAAAACC

P1-G10 CGTCACTTGC TCTGGAGATGCATTGGGACAAAAATATGCTTC TGGTATCAACAGAAGCC

FR2-L CDR2-L

D2-57 AGGGAAAGCCCCTAAGGCCCTGATCTAT GCTGCATCCAGTTGCAAAGT GGGGTCCCATC

C1-54 TGGCAGGCTCCAGACTCCCTGCTAT GGTGCATCCACTAGGGCCACT GGTGTCCCAGC

P1-G10 AGGCCAGTCCCCGTACTGGTCATCTT CAAGATTCCAAGCGGCCCTCA GGGATCCCTGA

FR3-L

D2-57 AAGGTTCAGTGGCAGTGGGCTGGGACAGATTCTACTCTCACCATCAGTAGTCTGCAACTTG

C1-54 CAGGTTCAGTGGCAGTGGGCTGGGACAGCGTTACTCTCACCATCGACAGCCTGCACTGCTG

P1-G10 GCGGTTCTCTGGCTCCAATTCTGGAACACAGCCACTCTGACCATCAGCGGGACCCAGGCTG

CDR3-L

D2-57 AAGATTTGCAACTTACTACTGT CAACAGAGTTACA.....GTACCCCCCTCG TTGGCC

C1-54 AAGATTTGCACTTATTACTGT CAGCAGTATAATAAGTGGCTCCGTACTCC TTGGCC

P1-G10 TGGATGAGGGCGACTATTATTGT CAGGCGTGGGACA...CTACAGCTTATGTC TTGGAA

FR4-L

D2-57 AAGGGACCAAGGTGGAAATCAA

C1-54 AAGGGACCAAGCTGGAGATCAA

P1-G10 CTGGGACCAAGGTACCGTCCTA

VARIABLE REGION - HEAVY CHAINS (Nucleic Acid)

FR1-H

D2-57 GAAGTTCAATTGTTAGAGTCTGGTGGCGGCTTGTTCAGCCTGGTGGTTCTTACGTCTT

C1-54 GAAGTTCAATTGTTAGAGTCTGGTGGCGGCTTGTTCAGCCTGGTGGTTCTTACGTCTT

P1-G10 GAAGTTCAATTGTTAGAGTCTGGTGGCGGCTTGTTCAGCCTGGTGGTTCTTACGTCTT

CDR1-H

D2-57 TCTTGCCTGCTTCCGGATTCACTTTCTCT CGTTACGTTATGTGG TGGGTTGCCAAGCT

C1-54 TCTTGCCTGCTTCCGGATTCACTTTCTCT CATTACGGTATGTCT TGGGTTGCCAAGCT

P1-G10 TCTTGCCTGCTTCCGGATTCACTTTCTCT CATTACTATGCAG TGGGTTGCCAAGCT

FR2-H

CDR2-H

D2-57 CCTGGTAAAGGTTGGAGTGGGTTCT TATATCTGGCCTTCTGGTGGCAATACTTATTAT

C1-54 CCTGGTAAAGGTTGGAGTGGGTTCT GTTATCTCTCTTCTGGTGGCCGTACTCTTTAT

P1-G10 CCTGGTAAAGGTTGGAGTGGGTTCT TATATCGTTCTCTGGTGGCAATACTTATTAT

	FR3 - H
D2 - 57	GCTGACTCCGTTAAAGGT CGCTTCACTATCTCTAGAGACAACCTAAGAATACTCTCTAC
C1 - 54	GCTGACTCCGTTAAAGGT CGCTTCACTATCTCTAGAGACAACCTAAGAATACTCTCTAC
P1 - G10	GCTGACTCCGTTAAAGGT CGCTTCACTATCTCTAGAGACAACCTAAGAATACTCTCTAC
	FR3 - H (contd)
D2 - 57	TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAG TAGCTAC
C1 - 54	TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAA
P1 - G10	TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAG AGGGACC
	CDR3 - H
	FR4 - H
D2 - 57	GATTTTTGGAGTAATGCTTTGATATC TGGGCCAAGGGACAATGGTCACCGTCTCAAGC
C1 - 54	..ACATTACTCCTACGCTATGGACGTC TGGGCCAAGGGACCACGGTCACCGTCTCAAGC
P1 - G10	...TATAACACCTCCCCTTGACTAC TGGGCCAGGGAACCTGGTCACCGTCTCAAGC

Exemplary ELISA data obtained with some of the described antibodies is as follows:

Table 2: Exemplary ELISA data

Isolate number	HA with Mg	HA w/o Mg	LA with Mg	LA w/o Mg
Control #1	0.15	0.10	0.13	0.07
C1-54	1.02	0.55	1.17	0.49
D2-57	0.68	0.09	0.13	0.07
P1-G10	0.87	1.06	0.16	0.08
Control #2	0.17	0.10	0.14	0.07
no phage	0.10	0.08	0.08	0.06
P1-G10, no cells	0.05	0.06	0.05	0.05
blank	0.05	0.05	0.05	0.05

HA indicates the high affinity open form. LA indicates the low affinity closed form of LFA-1. Control #1 refers to a phage that binds to a different target. Control #2 is another protein that binds to a different target.

The following is a comparison of the D2-57 light chain with a germline sequence. An antibody can include a D2-57 light chain with one or more of the following

substitutions, e.g., one, two, three, four, five, or six of the following substitutions (or insertion), e.g., at positions: S30G, L40P, A46L, L80P, W96ins, and S97T. For example, the antibody can include an insertion that provides W96.

D2-57 LC (top) VKI-02 :: JK1 (bottom)

FR1.....	CDR1.....	FR2.....	CDR2...
QDIQM T QSPSSLSASVGDRVTITC	RASQSIGSYLN	WYQQKTGAKP KALIY	AASSLQS
- - - - -S-----	- - - - -	- - P - - L - -	- - - - -
1 5 1 1 2 2 2 3 5 0	3 4 4	5 5	5 5
0 5 0 3 5 0	5 0 5	0 5	0 5
FR3.....	CDR3...	FR4.....	
GVPSRFSGSGSGTDFTLTISLQL E D FAT VYYC	QQSYSTP	SFGQQ G TKV E I KRT	
- - - - -P-----	- - - - -	WT-----	
5 6 7 7 8 8 8 9 9 9	9 1		
7 0 5 0 5 0 5 8 0 5	6 0		
	0		

S30G, P40L, L46A, P80L, W96delta, T97S

The following is a comparison of the C1-54 light chain with a germline sequence. An antibody can include a C1-54 light chain with one or more of the following substitutions, e.g., between one and eleven, two and five, or six and eleven of the following substitutions (or deletion), e.g., at positions: D1E, Q3V, V19A, T24R, D30S, V48I, V58I, A70E, D76S, K93N, and P95aΔ.

C1-54 LC (top) VKIII-L2 (bottom) :: JK2

FR1.....	CDR1.....	FR2.....	CDR2...
QDIQM T QSPATL S VSPGERVTLSC	TASQSVDSNL A	WYQQKPG Q APRL L IVY	GASTRAT
E-V-----A-----R-----S-----	- - - - -	- - I - -	- - - - -
1 5 1 1 2 2 2 3 5 0	3 4 4 4 5 5	5 9 0 5	5 5
0 5 0 3 5 0	5 0 5	0	0
FR3.....	CDR3...	FR4.....	
GVPARFSGSGSGTAF T LTIDSLQ E DFAVYYC	QQYNKWP	PYS F GQGT K L E I KRT	
- I ----- E ----- S -----	- - N --	- - T - -	
5 6 7 7 8 8 8 9 9 9 1			
7 0 5 0 5 0 5 8 0 5 6 0			
	0		

E1D, V3Q, A19V, R24T, S30D, I48V, I58V, E70A, S76D, N93K, P95a(insert)

An antibody can include a P1-G10 light chain with one or more of the following substitutions, e.g., between one and twelve, two and five, or six and twelve of the following substitutions (or insertion), e.g., at positions: Q1S, S2Y, V3E, V2I1, A28K, Q31aD, S34C, F49Y, V81M, T93S, T94S, and A95a(ins).

>P1-G10 LC(top) VL2-1 aka VL3 11-7 3r::JL1(bottom)

GSVLTQPPSVSVSPGQTASVTC	SGDALGQKYAS	WYQQKPGQSPVVLVIF	QDSKRPS		
SYE-----I--	---K-D---C	-----Y-----			
1 5 91 1 2 2 22223333333	3 4 4 4 5 5				
1 5 0 3 45780111234	5 0 5 9 0 5				
ab					
<hr/>					
GIPERFSGNSGNATATLTISGTQAVDEADYYC	QAWDTTA-	YVFGTGTKVTVL			
-----M-----	-----SS-A-----	-----	(JL1)		
5 6 6 7 7 8 8 8 9 9 9 9					
7 0 5 0 5 0 5 8 0 5 6 8					

S1Q, Y2S, E3V, I21V, K28A, D31aQ, C34S, Y49F, M81V, S93T, S94T, A95a(delta)

Any vg3-23 related heavy chain can include one or more of the following substitutions in the JH region:

vg3-23 (top) vs. GLG 3-23 (bottom)

EVQLLESGGG	LVQPAGSLRL	SCAASGFTFS	xYxMxWVRQA	PGKGLEWVSX
-----	-----	-----	S-A-S-----	-----A
IxxSGGxTxY	ADSVKGRTI	SRDNSKNLY	LQMNSLRAED	TAVYYC
-S-Q----S-Y-	-----	-----	-----	-----

JHs

JH1	---AEYFQHWGQGTLVTVSS	
JH2	---YWYFDLWGRGTLVTVSS	
JH3	-----AFDIWGQGTMVTVSS	
JH4	-----YFDYNGQGTLVTVSS	FDYWGQGTLVTVSS
JH5	---NWFDPWGQGTLVTVSS	
JH6	YYYYYGMDVWGQGTTVTVSS	

An antibody can include a D2-57 heavy chain with one or more of the following substitutions, e.g., between one and eleven, two and five, or six and eleven of the

following substitutions, e.g., at positions: R31S, V33A, W35S, Y50A, W52S, P52aG, N56S, S94R, A99Y, I102Y, and M108L.

D2-57 HC (top) V3-23 :: JH4 (bottom)

FR1.....	CDR1.	FR2.....	CDR2..
EVQLLES GGG LVQPGGS LRL SCAASGFTFS	RYVVMWVRQA	PGKGLEWVS V	
-----	S-A-S-----	A-----	
1 5 1 1 2 2 2	3 3 3 3	4 4 5	
0 5 0 2 5	0 1 3 5	0 5 0	
CDR2.....FR3.....	CDR3.....		
ISPSGGRTLY ADSVKGRFTI SRD N SKNTLY LQMNSLRAED TAVYYCAS SYDFWSN			
-SG--S-----R			
5 55 5 5 6 6 6 7 7 7 8 888 8 9 9 9 9 999999			
1 23 5 9 0 5 9 0 5 9 0 222 5 0 2 4 678888			
a	abc	abc	

CDR3 FR4.....			
AFDI WGQGTMVTVSS			
Y--Y -----L-----			
91 1 1			
90 0 0			
0 3 8			

S31R, A33V, S35W, A50Y, S52W, G52aP, S56N, R94S, Y99A, Y102I, L108M

An antibody can include a C1-54 heavy chain with one or more of the following substitutions, e.g., between one and seven, e.g., one, two, three, four, five, six, or seven of the following substitutions, e.g., at positions: H31S, G33A, V50A, P52aG, R56S, L58Y, and K94R.

C1-54 HC V3-23 :: JH6

FR1.....	CDR1.	FR2.....	CDR2..
EVQLLES GGG LVQPGGS LRL SCAASGFTFS	HYGMSWVRQA	PGKGLEWVS V	
-----	S-A-----	A-----	
1 5 1 1 2 2 2	3 3 3 3	4 4 5	
0 5 0 2 5	0 1 3 5	0 5 0	
CDR2.....FR3.....	CDR3.....		
ISPSGGRTLY ADSVKGRFTI SRD N SKNTLY LQMNSLRAED TAVYYCAK HYSYA			
--G--S-Y-----R			
5 55 5 5 6 6 6 7 7 7 8 888 8 9 9 9 9 9999			
1 23 5 9 0 5 9 0 5 9 0 222 5 0 2 4 6788			
a	abc	a	

MDV WGQGTTVTVSS
YYYYYG-----

1	1
0	0
0	3

S31H, A33G, A50V, G52aP, S56R, Y58L, R94K

An antibody can include a P1-G10 heavy chain with one or more of the following substitutions, e.g., between one and seven, e.g., one, two, three, four, five, six, or seven of the following substitutions, e.g., at positions: H31S, S33A, Q35S, Y50A, G52S, S52aG, and N56S.

P1-G10 HC JH4

FR1.....	CDR1.	FR2.....	CDR2...
EVQLLESGGG	LVQPGGSLRL	SCAASGFTFS	HYSMOWVRQA
-----	-----	-----	PGKGLEWVSY
1 5 1 1 2 2 2	-----	3 3 3 3	4 4 5
0 5 0 2 5	-----	0 1 3 5	0 5 0
<hr/>			
CDR2.....	FR3.....	CDR3.....	
IGSSGGNTYY	ADSVVKGRFTI	SRDNSKNTLY	LQMNSLRAED
-SG--S-----	-----	-----	TAVYCAR
5 55 5 5 6 6 6 7 7 7 8 888 8	-----	9 9 9 9	999999
1 23 5 9 0 5 9 0 5 9 0 222 5	-----	0 2 4	678888
a	abc	abc	
<hr/>			
FDYWGQGTLVTVSS			
Y-----			
1			
0			
3			

S31H, A33S, S35Q, A50Y, S52G, G52aS S56N

Other embodiments of the invention are within the following claims.

WHAT IS CLAIMED:

1. A protein comprising an immunoglobulin heavy chain (HC) variable domain sequence and an immunoglobulin light chain (LC) variable domain sequence, wherein the HC variable domain sequence and the LC variable domain sequence form an antigen binding site that binds to activated LFA-1, and the LC variable domain sequence comprises the light chain of D2-57, and the HC variable domain sequence comprises the heavy chain of D2-57.

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